ol. 94 Part 1 March 1953

The Quarterly Journal of Microscopical Science

(Third Series, No. 25)

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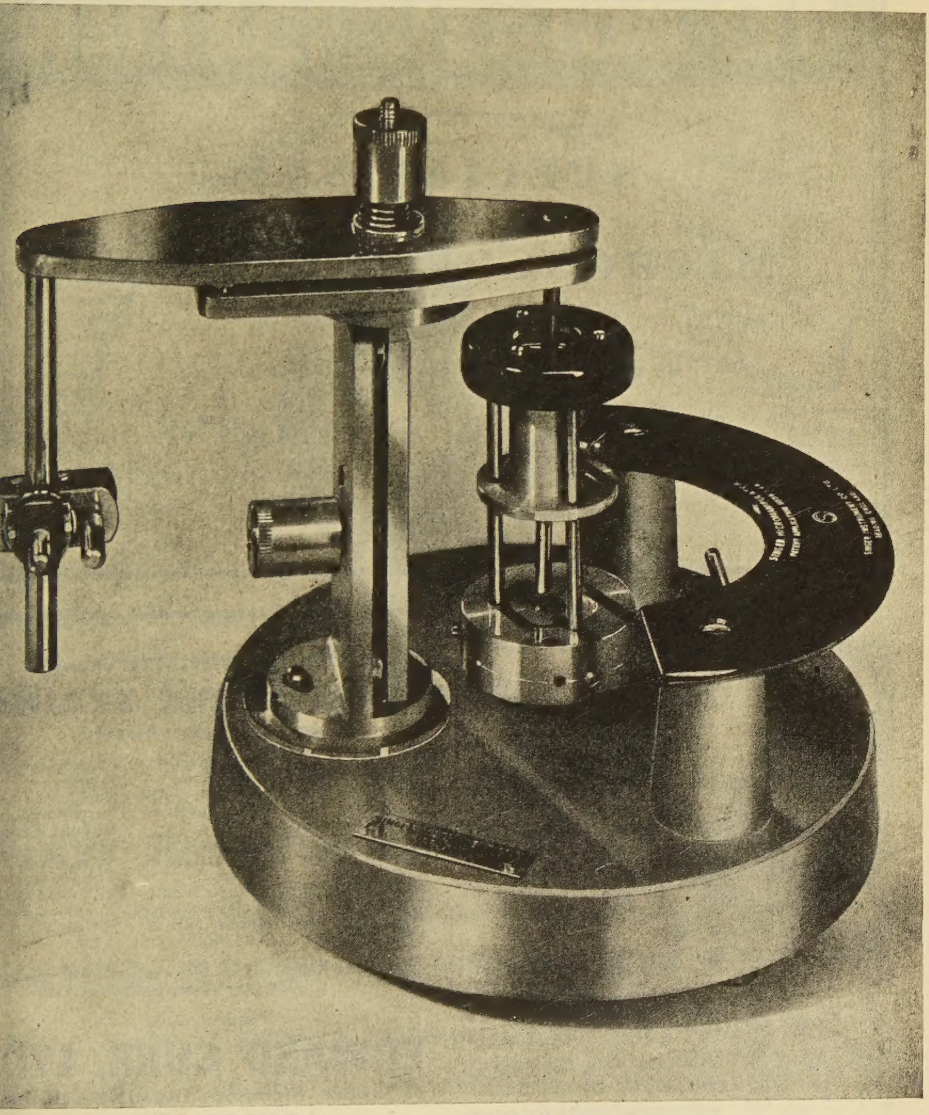

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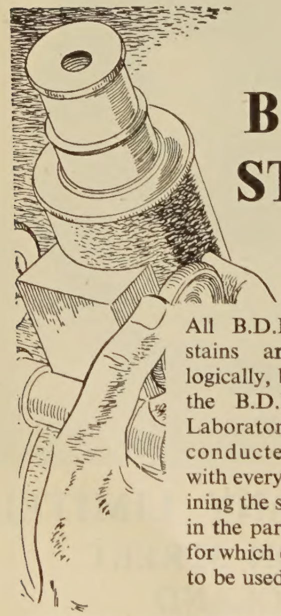

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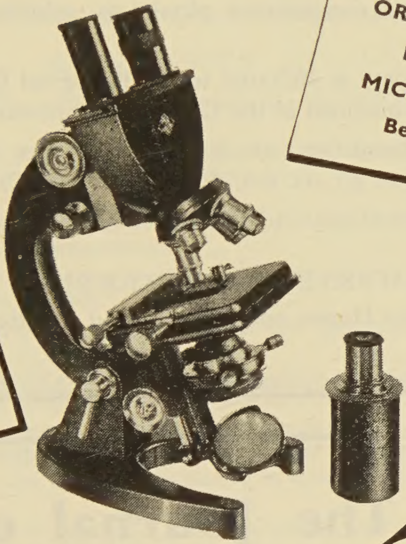

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¶ The current issue is Volume 30, No. 1, March 1953.

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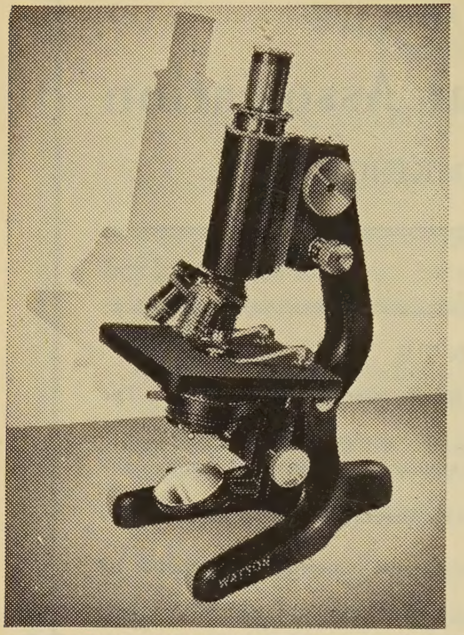

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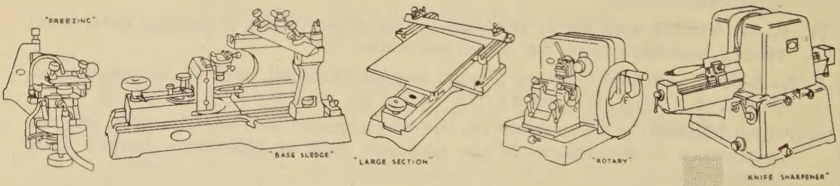

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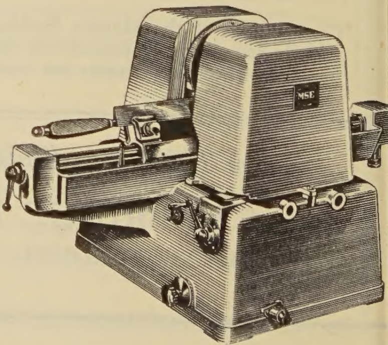

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The Use of Basic Dyes and Ribonuclease for the Cytochemical Detection of Ribonucleic Acid[1]

By J. BRACHET

(*From the Laboratoire de Morphologie animale, Faculté des Sciences de l'Université libre de Bruxelles*)

SUMMARY

1. Basic dyes (methyl green/pyronin, and toluidin blue), in conjunction with ribonuclease, may be used to detect the cytochemical localization of ribonucleic acid. This technique has been examined critically.

2. The fixative used is important and results vary with different materials. It is suggested that Zenker and Serra fixation should be compared in preliminary investigations. Unexplained anomalies arise in the use of frozen-dried material.

3. Details are given of the methods of use of ribonuclease and of the dyes, and a general technique is given for preliminary investigations.

4. It is concluded that there is a general parallelism between intensity of staining with basic dyes and content of ribonucleic acid, but there is no evidence yet available to justify the use of such staining for the quantitative estimation of ribonucleic acid.

INTRODUCTION: PRINCIPLE OF THE METHOD

IN 1940 I discussed the possibility of detecting ribonucleic acid cytochemically, by a method based on its affinity for basic dyes and its specific digestion by ribonuclease. During the subsequent 12 years this technique has been used extensively and appears generally to have given satisfactory results. Several improvements and alterations of the initial technique have appeared, and it seems useful to examine the requisite conditions for obtaining the best results: I shall then discuss critically the chief causes of error which may be encountered in this method.

The principle of the method is simple: nucleic acids, on account of their high acidic character, combine easily with basic dyes (see Michaelis, 1947, for a recent and competent discussion of this question). Thus, nucleic acids and nucleoproteins are basiphilic compounds during the common histological techniques. In fact, it is probable that, *in situ*, most other basiphilic substances disappear during histological procedures: acid soluble compounds are extracted and we have shown with Shaver (1948) that an extraction from 15 to 30 minutes with ice-cold 5–10 per cent. trichloracetic acid does not diminish basiphilia. Fatty acids which could have been a cause of error (Holtfreter, 1946) need not be taken into account when fixing agents are used which do not preserve lipides *in situ*: alcohol and toluene used during embedding and staining result in a sufficient elimination of lipides. Brachet and Shaver (1948) and

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[1] Prepared for the Cytochemical Commission of the Society for Cell Biology.

[Quarterly Journal of Microscopical Science, Vol. 94, part 1, pp. 1-10, Mar. 1953.]

more recently Panijel (1951*a*) have further shown that a prolonged extraction of the sections by boiling alcohol-ether mixture in a Soxhlet apparatus does not affect basiphilia.

In fact, the only basiphil substances rather frequently present in cells, and which might be confused with nucleic acids, are the glycoproteins, easily recognized by their metachromasia, and the phosphoproteins, responsible for the discrete basiphilia of the yolks of many eggs.

It is with the aim of providing the staining with basic dyes with the specificity it lacked that we were led (1940, 1942) to use ribonuclease to digest the ribonucleic acid present in sections: after the action of this enzyme, structures containing nucleic acid lose their affinity for basic dyes.

Desoxyribonucleic acid may also be removed by desoxyribonuclease, but the usefulness of this enzymatic method is secondary because the Feulgen reaction provides an elegant and probably faithful method for detecting this nucleic acid. The usefulness of desoxyribonuclease, however, gives us the possibility of checking the results obtained by the Feulgen reaction. This is especially useful when one deals with an atypical (e.g. cytoplasmic) localization of Feulgen positive granules.

It is important to mention that the idea of combining a nuclease digestion with the use of basic stains is far from new. In 1913, van Herwerden (1913, 1914) applied this principle to numerous animal tissues. If the results obtained by this author and her techniques have been forgotten, it is because the enzyme used was of low activity and non-specific: it was active with both types of nucleic acids and incubation had to last for several days. It was a mixture of enzymes, of no great cytochemical value. The interpretation of her results was especially doubtful owing to the fact that we had at that time the erroneous idea that ribonucleic acid was only found in plants and in pancreas; one had to wait until the years 1930–40, and for data similar to our demonstration of the presence of ribonucleic acid in the cytoplasm of sea urchin eggs (Brachet, 1933), to be able to interpret accurately the results of van Herwerden.

To give full reliability to the nuclease method of detection of nucleic acid, it is essential that only highly purified enzymes should be used. This has become possible since the work of Dubos and Thompson (1938), Dubos and McLeod (who were the first to use ribonuclease to detect ribonucleic acid in bacteria, 1938), and especially that of Kunitz (1940, 1950), who succeeded first in crystallizing ribonuclease, then desoxyribonuclease.

It is on account of its highly specific enzymatic action that the use of ribonuclease must be preferred to that of hot normal hydrochloric acid (Vendrely-Randavel, 1949) and of 10 per cent. perchloric acid at 4° C. for 18 hours (Erickson, Sax, and Ogur, 1949). These acids effectively extract ribonucleic acids from the sections, but there is no proof that this action is selective. They bring about a depolymerization and even a liberation of part of the purines of desoxyribonucleic acid, a loss of affinity of this acid for methyl green, a lessening of U.V. absorption, and also the Feulgen reaction becomes positive, which is a disadvantage for quantitative work.

In the following pages the discussion will be confined to that method of detection of nucleic acids by basic dyes which is the most useful: the detection of ribonucleic acid with ribonuclease and methyl green/pyronin mixture or toluidin blue.

PRELIMINARY TECHNICAL CONSIDERATIONS

The chief points in question are the choice of the fixing agent, the determination of the optimal conditions for enzymatic action, and the choice of the dye. These problems were discussed in detail by Stowell and Zorzoli (1947) and more recently by Taft (1951*a*); their conclusions agree very satisfactorily with our personal experience with the method.

Choice of the fixative. The best results are obtained after fixation with acetic Zenker solution, Carnoy solution (6 p. alcohol, 3 p. chloroform, 1 p. acetic acid) and with the alcohol/formalin/acetic acid (6:3:1) mixture of Serra (1946). Zenker solution generally gives better fixation, but has the disadvantage of rendering ribonucleic acid particularly resistant to the action of ribonuclease: the enzyme has to be used in concentrated solution and for a considerable time to be effective, and complete digestion of ribonucleic acid cannot be obtained in the case of some organs after the use of this fixative. These difficulties are probably due to the presence of a mercuric salt ($HgCl_2$) in the Zenker solution which forms complexes of low solubility with nucleic acids; the sections may retain, even after washing, sufficient $HgCl_2$ to inhibit ribonuclease.

Serra's solution gives an excellent staining with basic dyes and allows a much better fixation than that of Carnoy, which may, however, be used successfully in special cases (eggs of insects or of *Ascaris*, for instance). However, the use of both these fixatives is not convenient in the case of material containing a cavity full of an aqueous liquid such as an amphibian blastula or gastrula; in such cases the cells are considerably distorted and fixing is very poor.

Ribonucleic acid of organs fixed with Serra or Carnoy solutions is easily digested by ribonuclease, but a new difficulty arises: under these conditions the nucleic acid of many organs is sufficiently soluble in water alone for a large proportion of the basiphilia to be washed away.

An important point, worth mentioning, is the following: whatever the fixative used, the time of fixation has to be short. When prolonged, basiphilia is progressively diminished, especially on the border of the sample of tissue, which has been in contact with the fixative for a longer time. The affinity of nucleic acids for basic dyes is also diminished when the fixed organs have been stored for too long in alcohol: it is essential, once fixing is completed, to embed the tissue in paraffin as soon as possible.

It is not yet known why nucleic acids lose their affinity for basic dyes after a prolonged contact with an acid fixative or alcohol. There is no loss of ribonucleic acid: unpublished observations of our co-worker Steinert have shown that batrachian eggs may be kept for weeks in alcohol without any loss of ribonucleic acid (determined by chemical methods).

One sees that it is impossible to give an ideal method of fixation applicable in all cases: one has to vary experimental conditions when a new material is investigated, and it will often be useful to compare the results of Zenker and Serra fixation. In both cases, however, the fixation must not exceed 1 hour for objects of 2–3 mm. in diameter, and no time should be lost before embedding in paraffin. It is probably due to lack of consideration of these two last points that Taft (1951*b*) has not obtained satisfactory results after acetic-Zenker or Serra fixation.

On the contrary, Taft (1951*b*) found preparations fixed by freezing and drying satisfactory. Our own experience, perhaps limited, has been less successful and we could never obtain really intense basiphilia after using this method of fixation and embedding. It is possible that the presence of lipides, which are not necessarily eliminated by this method, accounts for failure to obtain good results: Monné (1946) has emphasized the advantage, for the study of basiphilia, of fixatives which are good lipides solvents (Carnoy or Serra solution, for instance). We have mentioned earlier that Holtfreter (1946) has shown that fatty acids may be responsible for part of the cytoplasmic basiphilia: this is probably why the staining of squashes simply fixed in 40 per cent. acetic acid gives much poorer results than those obtained after embedding in paraffin (methyl green-pyronin staining).

Choice of the enzyme. Since Kunitz (1940) succeeded in crystallizing ribonuclease, the crystallized enzyme has become the substance of choice for these studies. Brachet used it successfully in 1942, and more recently, M. R. McDonald (1948) has described a technique for preparing crystallized ribonuclease, completely free from proteolytic activity. This enzyme has been used with good results by Kaufman, McDonald, and Gay (1951), which are in agreement with our previous observations (1942).

Jacobson and Webb (1952) have found that traces of proteolytic enzymes present in desoxyribonuclease may be efficiently inhibited by addition of glutathione or hydroxylamine. It would probably be advantageous to use these inhibitors with ribonuclease when the presence of traces of proteolytic enzymes is suspected.

The work and discussion of Kaufman and others, and of Schmidt, Cubiles, and Thannhauser (1951), shows that our understanding of the mode of action of ribonuclease, even in the case of ribonucleic acid solution, is far from clear. In particular, there appear to exist several distinct ribonucleases, having different specificities. It would certainly be of great interest to know more of the action of these enzymes, and to try purified samples on sections of various organs. Furthermore, it is evident that we know hardly anything about the changes suffered by nucleoproteins and nucleic acids themselves when in contact with such drastic agents as histological fixatives.

The optimum concentration of ribonuclease and the best medium for making solutions has been the object of several investigations (Stowell and Zorzoli, 1947; Taft, 1951*b*; Kaufman, McDonald, and Gay, 1951; Brachet and Shaver, 1948, &c.). Here again the nature of the fixative and of the organ to be studied have to be taken into account. For a first trial, 0·1 mgr. of crystalline ribonuclease to each ml. of distilled water (adjusted to pH 6) should be tried. If this does not work, which may happen after fixation with Zenker solution, the enzyme concentration may be raised to 1 mgr./ml.; in a very unfavourable case (fixation with Flemming's solution), Kaufman, McDonald, and Gay (1951) had to use even higher concentrations—up to 6 mgr. enzyme to each ml.

The duration and temperature of hydrolysis may also be varied: maximum activity for ribonuclease occurs around 65° C., but, at that high temperature, distilled water alone extracts a considerable fraction of the ribonucleic acid of numerous organs. Most authors, including ourselves, usually hydrolyse for 1 to 2 hours at 37° C.

It is important to control the specificity of the enzymatic digestion by similar treatment of a control section with distilled water (adjusted to pH 6) during the same time and at the same temperature. As we have seen, the extraction of ribonucleic acid by distilled water at 37° C. depends on the fixative used and the organ studied.

Choice of the dye. Most often methyl green/pyronin mixture (Unna, 1902) or toluidin blue are used for staining the sections. But other basic dyes, alone or mixed with other dyes, may be just as useful or even better (Giemsa, Flemming's tricolor stain, chromogallocyanin of Lagerstedt, 1949, &c.). Here discussion will be limited to the use of toluidin blue and of Unna's solution, the only cases in which I have an extensive personal experience.

Toluidin blue has the great advantage of being easy to use, the basic dye being utilized alone. It gives very accurate images when cytoplasmic ribonucleic acid is not concentrated (for instance, in the case of amphibian eggs at the early stages of development), but it is not adequate in cases where the tissues contain much ribonucleic acid, and it does not enable any distinction to be made between the two types of nucleic acids present in the nucleus.

The method of Unna, as we have shown (1940, 1942), tends to stain in green the desoxyribonucleic acid of the chromatin and in red the ribonucleic acid of the nucleoli and of the cytoplasm. It is, however, the use of ribonuclease which determines the specificity of the staining. The reasons why methyl green is selectively fixed to desoxyribonucleic acid are far from clear. However, a very recent study by Taft (1951*a*) has confirmed that, when favourable conditions are chosen, the Unna staining is the method of choice for detecting the localization of both types of nucleic acid in the cell.

Several formulae have been suggested since that of Unna (1902), which has a major disadvantage in the fact that the staining obtained, and especially with pyronin, is excessively labile during alcoholic differentiation. This step of the method requires practice and skill. A systematic investigation of the problem has recently been undertaken by Taft (1951*b*) who suggests the following solution: 0·5 gr. methyl green (purified by chloroform extraction) is dissolved in 100 c.c. of M/10 acetate buffer pH 4·4. The solution is again repeatedly extracted with chloroform until methyl violet is completely eliminated; finally 0·2 gr. of pyronin B are dissolved in the solution.

The mixture I prefer (see below) is not very different from the solution proposed by Taft (1951*b*). It has the advantage over Unna's formulae of giving stainings which are more stable towards alcohol; the stained preparation may actually be left from 5–10 minutes in 95 per cent. alcohol without any harm.

An important point for obtaining results with Unna's staining is the quality of the dyes used. Taft (1951*b*) has tried quite a number of stains, mostly of American origin: his conclusions may be found in his paper. Personally, we have obtained satisfactory results with methyl green produced by Anachemia, Grübler, and Ciba, as well as with pyronin from Geigy and Grübler. It is obvious that one has to eliminate all the methyl violet by repeated extraction in chloroform, whatever methyl green is used; the mixture may not be kept for more than from 2 to 3 weeks at ordinary temperature because an appreciable amount of methyl violet will be formed at the expense of methyl green.

TECHNIQUE GENERALLY USED

As we have just seen, it is not possible to give absolute rules concerning the procedure: the mode of fixation, the solubility of the ribonucleic acid of the organ to be studied and the activity of the enzyme available are all possible variables.

It is thus for general information that the following procedure is given. It is a compromise between the different possibilities among which the investigator may choose.

(*a*) Fixation with Zenker (containing 5 per cent. acetic acid) or Serra solution (alcohol/formalin/acetic acid 6:3:1). The time of fixation must not exceed 1 hour for objects of 2–3 mm. in diameter.

After Zenker's solution: wash with tap water for 24 hours, dehydrate and embed immediately in paraffin (through alcohol and toluene).

After Serra's solution: wash with 95 per cent. alcohol, 2–3 times; absolute alcohol, toluene, paraffin.

(*b*) Place the sections alternately on three slides, A, B, and C; deparaffinize and rehydrate by the usual procedure.

Slide A is stained immediately (see below).

Slide B is placed for 1 hour at 37° C. in distilled water, adjusted to pH 6·0, then stained (see below).

Slide C is placed for 1 hour at 37° C. in a solution of crystalline ribonuclease (Kunitz) containing 0·1 mgr. enzyme to each ml. distilled water, adjusted to pH 6·0, then stained (see below).

(*c*) Staining:

(i) Unna's solution.

The solution has the following composition:

Methyl green (purified with chloroform)	0·15	gr.
Pyronin B.	0·25	gr.
Alcohol 95 per cent.	2·5	ml.
Acetate buffer pH 4·7 to final volume of:	100	ml.

Stain for 20 minutes, wash rapidly with distilled water, differentiate with 95 per cent. alcohol for 5–10 minutes, absolute alcohol, toluene, balsam.

(ii) Toluidin blue (chiefly used for early embryonic stages). Stain for 20 minutes in a saturated aqueous solution of toluidin blue (Ciba) (approximately 2 per cent.); wash rapidly in distilled water, differentiate twice successively in 95 per cent. alcohol, toluene, balsam.

DISCUSSION OF THE METHOD

It is obvious that the specificity of the method is dependent on the well established specificity of the enzyme: Dubos and Thompson (1938) had already shown that the enzyme has no detectable effect on different proteins, glyco-proteins, polysaccharides, hexosephosphates, peptones, lipides and desoxyribonucleic acid. We have also observed (1942) that no phosphorus is liberated from phosphoproteins.

However, Cohen (1945) and Schneider (1946) have shown that different samples of ribonuclease may be contaminated by various amounts of proteases, but it has been established (Brachet and Shaver, 1948) that these proteolytic impurities are not sufficiently active to diminish, to any appreciable extent, the staining reaction of protein-bound arginin and tyrosin, when sections are treated for 1 hour at 52° C. by ribonuclease; in contrast, the disappearance of basiphilia is complete under the same conditions. The recent demonstration by Kaufman, MacDonald, and Gay (1951) that ribonuclease completely free from proteases, as prepared by McDonald (1948), gives exactly the same results as the ones we described previously (1942) removes all remaining doubts concerning specificity.

We must not forget that the results obtained by our method are in complete agreement with those of Caspersson (1940, 1941) obtained by a completely different method (U.V. absorption spectrophotometry). It is also known that these cytochemical data are in excellent agreement with results of direct micro-determinations of ribonucleic acid, which have been performed on numerous organs (Brachet, 1941; Norberg, 1942; Davidson, 1947). These are additional arguments supporting the validity of the method.

There also do not seem to be any serious reasons for doubting the value of the localization found: here again, results agree with those published by Caspersson (1941).

One may thus consider the method as satisfactory if one's needs are limited to the qualitative detection of ribonucleic acid inside the cell and if one takes into account the different causes of error we mentioned, concerning the choice of fixative, the enzyme, and the dye.

Is one, however, allowed to go further, and is it justifiable to use the method on a quantitative basis with the aid, for instance, of a microspectrophotometer for determinations of the amount of dye bound to the various cellular structures? It is true that the intensity of the pyronin staining of the cytoplasm is a useful indication of the amount of ribonucleic acid present. To be convinced one only has to remember the parallelism observed, in a series of organs, when the values for chemical determination of ribonucleic acid content (Brachet, 1941) and the estimation of basiphilia are compared. This parallelism is, however, approximate and one must not expect more than semi-quantitative results.

One must be much more careful if one considers carrying out quantitative determinations and using microspectrophotometric techniques for ribonucleic acid determinations. Attempts made with this aim by Pollister and Ris (1947) have been criticized by Stowell and Zorzoli (1947), who have emphasized the difficulties already mentioned earlier, due to fixation and to solubility or ribonucleic acid itself in distilled water. Moreover, Kurnick (1950) and Kurnick and Mirsky (1950) have shown that, whereas desoxyribonucleic acid (when polymerized) combines stoichiometrically with methyl green, the complexes of ribonucleic acid and pyronin are much less stable. While one can determine satisfactorily the quantity of polymerized desoxyribonucleic acid of the nucleus by measuring the amount of bound methyl green (Kurnick, 1950), the same cannot be done with ribonucleic acid and pyronin. Besides, in the method Kurnick (1950) used successfully, the staining is obtained by the use of methyl green alone to avoid complications due to the pyronin in Unna's solution. In fact, we do not think it would be safe or advisable to try to use Unna's method of staining for quantitative determinations of intracellular nucleic acid, so long as the mechanism of this procedure is not better understood. The interpretation advanced by Pollister and Leuchtenberger (1949), Kurnick (1950), Kurnick and Ris (1950) has not been completely confirmed by Taft (1951*a*). For this last author, the affinity of methyl green for desoxyribonucleic acid cannot only depend on its degree of polymerization and he thinks 'the presence of orthophosphoric groups can hardly be the mechanism for this stain'. Certain indirect results, obtained in Brussels by M. Errera and by L. Lison, are also difficult to explain on the basis of the existing theories concerning the mechanism of the Unna staining. Sibatani (1952), Alfert (1952), Kurnick (1952), and Chayen (1952) have also drawn attention to the complexities of the processes involved in the use of this differential stain.

It thus appears that if the quantitative determination of desoxyribonucleic acid by staining with methyl green alone (Kurnick, 1950) is possible, it is preferable, if one wants to determine ribonucleic acid in the cytoplasm, to use less complicated staining techniques than the one used by Unna: one may think of toluidin blue (Jeener and Brachet, 1944; Hydén, 1943) and of chromogallocyanin (Lagerstedt, 1949), both of which follow Beer's law more faithfully.

Finally, it is important to emphasize one more point. It is known that ribonucleic acid is found in cells, bound to very diverse constituents: while an important fraction of this acid is bound to small particles (the microsomes), mitochondria contain some also, and furthermore, especially in growing tissues, an important fraction of ribonucleic acid is in the shape of a low molecular weight nucleoprotein which cannot be sedimented by ultracentrifugation (Brachet and Jeener, 1944; Claude, 1943). We know almost nothing about the affinity of these various fractions for basic dyes after fixation and embedding by the usual histological methods. The small number of facts known (Panijel, 1951*b*) seem to indicate that a structure is basiphilic only if it contains a high concentration of ribonucleic acid: thus, yolk platelets, although they do contain an appreciable amount of ribonucleic acid, are only slightly basiphilic (Panijel, 1951*b*). It could therefore be, as Panijel (1951*b*) suggested, that basiphilia of histological sections is exclusively due to microsomes. It is conceivable that structures containing less ribonucleic acid are not sufficiently basiphilic to be distinguished by the methods used and that low molecular weight ribonucleoproteins are lost during fixation and embedding. An experiment of Cleland (1950) seems to indicate that only alkaline phosphatase bound to ultracentrifugeable particles is detectable by Gomori's method and the same might be true for nucleoproteins.

This point is of importance for the interpretation of results obtained by cytochemical investigations of ribonucleic acid, and will only be answered by careful quantitative studies.

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On the Cuticle of the Scorpion *Palamneus swammerdami*

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SUMMARY

1. The cuticle of the scorpion *Palamneus swammerdami* shows the same fundamental layers as that of insects. It consists of a thin outer epicuticle and a thick inner endocuticle; the outer layers of the latter are transformed by hardening into an amber-coloured exocuticle.

2. The chemical characteristics of the constituent layers of the cuticle in different regions are described and it is shown that the proteins impregnating the endocuticle during hardening appear to be different from those described in insects and possess resistant properties.

3. The exocuticle in addition to giving evidence of tanning indicates the presence of disulphide bonding. A similar indication is obtained in the epicuticle.

4. The mode of hardening of the cuticle of scorpion is discussed and compared with similar processes in other arthropods.

INTRODUCTION

SINCE the time of Yonge's work (1932) on the cuticle of *Homarus*, much evidence has been brought forward to show that the two fundamental layers, epicuticle and endocuticle, are common to the cuticle of all insects and Crustacea (Drach, 1939; Pryor, 1940*b*; Dennell, 1947*b*; Krishnan, 1951). These two layers stain red and blue respectively in Mallory's stain. It has been suggested that this pattern may be characteristic of the cuticle of all arthropods, but Blower (1951) has reported the absence of a layer homologous to the epicuticle in myriapods, as well as certain chemical peculiarities in the endocuticle in this group. A similar difference in the cuticular constitution of the scorpion *Buthus europaeus* and *Buthus australis* has been emphasized by Lafon (1943*b*), who observed that the outermost layer which he believed might correspond to the epicuticle of insects showed a chemical constitution different from that of cuticulin and unlike the latter was resistant to the action of alkalis. However, these differences were not correlated with the histological features of the cuticle in regard to which in Arachnida there is little information. Browning (1942) described in *Tegenaria atrica* only two layers in the cuticle, an amber-coloured exocuticle and an inner colourless endocuticle, but Cloudsley-Thompson (1950*a*, 1950*b*), noted in arachnids and myriapods studied by him, in addition to the above two layers, a colourless epicuticle. In this connexion it is of interest to observe that Langner (1937) described in Diplopoda an outermost thin colourless layer which she called the epicuticle, but Blower (1951) doubts the histological validity of the layer, considering it

[Quarterly Journal of Microscopical Science, Vol. 94, part 1, pp. 11-21, Mar. 1953.]

a diffraction effect. In view of such contradictory results, it would appear that there is considerable uncertainty as regards the existence of an epicuticle in arthropods other than insects and Crustacea, and it was therefore felt that a detailed examination of the cuticle of a common arachnid was warranted to gain a more complete picture of the arthropod cuticle.

The material used in the following investigation consisted of scorpions belonging to the species *Palamneus swammerdami*, which occurs in abundance in south India. Paraffin, frozen, and hand sections of the cuticle were studied. For paraffin sections the cuticle was double-embedded in celloidin and paraffin. The stains successfully used were Mallory, Heidenhains haematoxylin, and Masson's trichrome stain. Frozen sections were prepared from material fixed in formalin and impregnated successively in 12½ per cent. and 25 per cent. gelatin. The histochemical tests were performed mostly on frozen sections. Hand sections and fresh material cut with a freezing microtome using sugar solution were also employed to confirm and extend the observations made above. The chemical reagents used for the detection of fats, phenols, proteins, and other constituents of the cuticle are mentioned in appropriate places in the text.

Structure and staining reactions

The cuticle of arachnids has so far received comparatively little attention. Previous work on this subject, apart from a few references to the scorpion cuticle, is confined to spiders (Browning, 1942; Cloudsley-Thompson, 1950*a*) and ticks (Lees, 1946, 1947). Lafon (1943*b*), in the course of a biochemical estimation of the integument of the scorpion, observed that the cuticle consists of a thin, outer, amber-coloured layer and a wide, inner, colourless layer formed of chitin. In spite of an apparent correspondence with the insect cuticle, he found that a dissimilarity in chemical constitution of the amber layer made it difficult to consider it homologous to the optically similar layer of the insect cuticle. A brief reference to the epicuticle of scorpions has been made by Cloudsley-Thompson (1950*a*), who, on the basis of a differential solubility in chlorated nitric acid, distinguished an epicuticle and a tanned exocuticle.

In the present study it has been observed that in the hardened cuticle such as occurs in the walking legs and the post-abdomen, there is distinguishable a wide colourless inner layer, the endocuticle, and an outer thin amber-coloured layer, the exocuticle, external to which is a very thin membranous layer which, as will be shown in the sequel, corresponds to the epicuticle (fig. 1). The endocuticle shows horizontal lamellae which are more prominent and farther apart in the inner regions and occur closer together distally. In the exocuticle such striations are hardly visible. In addition vertical striae extending through the endocuticle and the exocuticle are distinguishable, recalling strongly the description of similar striations in *Tegenaria* (Browning, 1942; Sewell, 1951) and *Ixodes* (Lees, 1946), in which they have been homologized with the pore-canals of insects. The amber-coloured exocuticle is approximately about one-eighth or one-tenth of the entire width of the cuticle. The thin outermost colourless layer recalls the description of a similar layer in Diplopoda (Langner, 1937; Blower, 1951), which has been interpreted as an epicuticle by Langner and as merely a diffraction effect by Blower. Traversing the cuticle are the prominent dermal gland ducts which arise from the hypodermis and open externally to the amber layer. The above-mentioned features are characteristic of all hardened parts although variations in extent of the constituent layers occur in different regions. In the sternites (fig. 2), the outermost colourless layer is thinner and the amber-coloured region is much attenuated. In the arthrodial membranes (fig. 3) there is no evidence of tanning and the layer corresponding to the outermost colourless layer is thrown into folds.

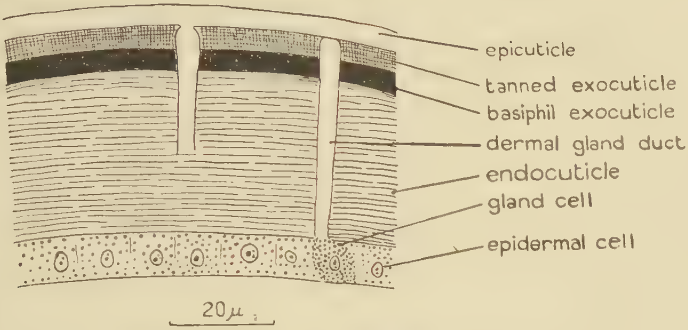

FIG. 1. Transverse section of the hardened cuticle of a leg. Mallory.

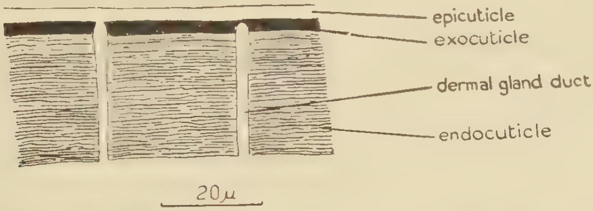

FIG. 2. Transverse section of sternite cuticle. Iron haematoxylin.

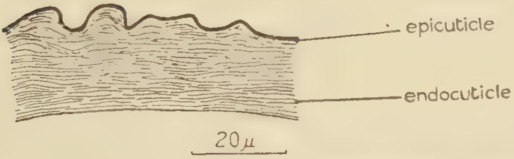

FIG. 3. Transverse section of arthrodial membrane. Iron haematoxylin.

In previous studies of the cuticle of insects and Crustacea it has been observed that the epicuticle always stained red and the endocuticle blue with Mallory's stain. That such differential staining of the epicuticle and endocuticle is not obtained in all arthropods is shown by Blower (1951), who observed that in myriapods it is the exocuticle which stains red with Mallory and otherwise shows a staining behaviour similar to that of the epicuticle of insects, as may be seen from a loss of staining property due to tanning of this region and a resumption of it after treatment with diaphanol. The endocuticle, though staining with the aniline blue of the Mallory stain, is definitely fuchsinophil in the sclerites of *Lithobius*. In the scorpion the endocuticle always stains blue and the exocuticle red with Mallory, but the exocuticle shows a loss of affinity to the stain with the assumption of an amber colour; so that as has been observed in *Sarcophaga* (Dennell, 1947*a*) and in myriapods (Blower, 1951), there appears to be an in-pushing of the red-staining zone. But unlike in *Sarcophaga* and other insects, the epicuticle is not here involved, for the thin layer overlying the exocuticle is not responsive to any of the stains used. With Heidenhain's iron haematoxylin the outermost colourless layer and the tanned part are unstained but the entire endocuticle stains blue. In the arthrodial membrane, which is not tanned, the epicuticle stains deep blue and the endocuticle is unstained or only very feebly stained. With Mallory the arthrodial membrane endocuticle takes up a blue colour and the epicuticle red. The above staining reactions suggest that the hardening of the epicuticle involves a loss of reactive properties to staining, with the result that in the hardened regions it is colourless and unstained. The absence of a staining reaction with haematoxylin in the endocuticle of the arthrodial membranes and its staining in the tanned cuticle might suggest that in the latter the endocuticle is impregnated with some substance responsive to haematoxylin. These changes recall strongly what has been observed in insects and myriapods during tanning of the cuticle. Pryor (1940*b*) suggested from chemical tests that the substance responsible for staining with haematoxylin in the insect cuticle may be a protein.

Endocuticle

The chemical properties of the endocuticle (Table 1) show agreement in many respects with those of the myriapods (Blower, 1951). That the principal constituent of the endocuticle is chitin is shown by the positive chitosan test. The endocuticle is dissolved by concentrated mineral acids and its susceptibility to acids is more marked in the untanned cuticle. From the staining reactions of the endocuticle described above, it may appear that it undergoes a change in its composition during the hardening of the cuticle, presumably by the addition of substances similar to those impregnating the exocuticle. Such a possibility is reflected in the differential staining with haematoxylin before and after the formation of the exocuticle. In the untanned regions such as the arthrodial membranes only the epicuticle takes up the stain and the endocuticle is feebly stained, but in regions where tanning has taken place, the entire endocuticle stains blue, suggesting the presence at this stage of

TABLE 1

Name of test	*Epicuticle*	*Exocuticle*	*Endocuticle*
Chitosan	−	+ + +	+ + + +
Concentrated nitric acid (solubility in)	−	+	+ + + +
Concentrated sulphuric acid (solubility in)	−	+	+ + +
Biuret	−	+ + +	+ +
Xanthoproteic	−	+ + +	+ +
Millón's	−	+ + +	+ +
Argentaffin	+	+ + +	+ +
$FeCl_3$	−	−	+* −
Alkaline lead acetate	+ +	+ + +	−
Sodium sulphide	+ +	+ + +	+
Thioglycollate (swelling in)	+ +	+	−
Fehling's solution	+ +	−	−
Sudan IV	+ + +	−	−

* Outer region only.

substances reactive to haematoxylin. That this substance may be of the nature of a protein may be inferred from chemical tests. The biuret test is positive, the entire endocuticle taking on a violet colour, the intensity of coloration being only a little less than that shown by the exocuticle. With Millon's reagent a gradation of colour varying from red to yellow was obtained. In the soft and untanned cuticle the above reactions were negative. These results might suggest that the endocuticle, which is formed primarily of chitin, gets impregnated during the hardening of the cuticle by a substance similar to that present in the exocuticle. This substance possesses in itself resistant properties. As has been already pointed out the endocuticle in a hardened region is less susceptible to the action of concentrated mineral acids than in soft cuticle. An idea of its resistance to the action of alkalis may be obtained from the observation that after several hours' heating in concentrated potassium hydroxide solution at 180° C., the substance impregnating the endocuticle is still present and reacts to haematoxylin staining. In the above features, a close parallel may be seen to the condition described in myriapods (Blower, 1951) wherein the endocuticle is said to be impregnated with a substance which is of the nature of a precursor to sclerotin and possesses resistant properties. As in the myriapods, the suggestion that the substance impregnating the endocuticle may be a precursor to sclerotin receives further support from the result of the argentaffin reaction in the endocuticle, which turns brown (though not as intensely as in the exocuticle), indicating a substance rich in phenolic groups. This observation is in conformity with the evidence of an inward extension of the tanned zone which is possible only if there is present in the endocuticle a substance which can be sclerotized. Further support is lent to this assumption by the positive ferric chloride reaction in the outermost parts of the endocuticle close to the tanned zone, suggesting the presence of dihydroxyphenols probably associated with the impending tanning of this region.

Exocuticle

Blower (1951) suggested that a criterion for differentiating the cuticular layers is their optical appearance. The unreliability of this criterion is shown by the optical similarity of the exocuticle of myriapods to the epicuticle of insects (see Cloudsley-Thompson, 1950*a*). Similarly, the amber-coloured layer in the scorpion cuticle apparently resembles in appearance the epicuticle of insects. Lafon (1943*b*) considered that the amber-coloured layer of the scorpion cuticle would be morphologically homologous with the cuticulin layer of insects. That it is undoubtedly an exocuticle formed by the tanning of the outer layer of the endocuticle is seen after treatment with concentrated potassium hydroxide. Pieces of cuticle heated in KOH at 180° C. for several hours and subsequently sectioned in paraffin show the amber-coloured layer in which a little of the coloration still persists, partially separated from the rest of the endocuticle. The horizontal lamellae characteristic of the endocuticle are now clearly seen and other structural features correspond fully with those of the endocuticle. Such sections when treated with iodine sulphuric acid give a deep violet coloration in the amber region, the colour being as intense as in the endocuticle. In view of the above results, it is clear that this layer corresponds to the exocuticle of insects.

The nature of the substance impregnating the exocuticle and responsible for the tanning of this region appears to be similar to that described in the insect cuticle. The positive xanthoproteic and biuret reactions may show that it is a protein (Table 1). That it is also rich in phenolic groups is seen from the positive Millon's test. The intense argentaffin reaction obtained in this region supports the view that aromatic substances enter into its composition. The similarity of these reactions with those observed in the insect and crustacean cuticle (Dennell, 1946, 1947*b*) suggest that the process of hardening of the scorpion cuticle may involve phenolic substances combining with the proteins of the exocuticle. As in insects, dihydroxyphenols are indicated in those regions where tanning is impending, as seen from a positive ferric chloride reaction in the outlying parts of the endocuticle. From the absence of a similar positive test with ferric chloride in the exocuticle, it seems reasonable to suggest that the dihydroxyphenols indicated in the regions destined to be tanned undergo further changes resulting in the sclerotization of the layer in question.

The steps in the process of sclerotization and the sources of materials involved are not known, although, following the analogy in insect and crustacean cuticle, it may be assumed that phenolic substances indicated in the cuticle may form primary valence cross-linkages with the protein of the exocuticle, thus hardening it by a tanning action. But the nature of the protein involved does not appear to be identical with that described in the insects. This may be inferred from the presence of sulphur, which is known to be absent from the insect cuticle (Pryor, 1940*a*). Sections of the hardened cuticle of the scorpion when treated with alkaline lead acetate solution showed a black precipitate in the exocuticle and epicuticle, thereby indicating the presence of cystine or cysteine sulphur. Additional chemical tests were performed to confirm the presence of sulphur in the cuticle. The cuticular substance was heated with sodium carbonate and potassium nitrate and after dissolving in warm water was filtered. The filtrate was acidified with hydrochloric acid and heated to boiling-point. On addition of barium chloride a white precipitate was formed, indicating the presence of sulphur (see Hawk, Oser, and Summerson, 1949). The occurrence of sulphur in the cuticular proteins of the scorpion is of interest as it is not known to occur in the insect cuticle; but Lafon (1943*a*) noted sulphur in the carapace of *Limulus*, in which it contributes to the stability and hardness of the protein in the cuticle. From what is already known of the sclerotization in the insect cuticle, it appears reasonable to presume that the process of hardening in the scorpion may differ from that in insects and may involve sulphur. The role of sulphur in the hardening process and its relation to tanning is not clear, but it is possible that quinones may react with bisulphide groups as readily as with amino groups (see Wigglesworth, 1948). The condition observed in the scorpion is not unlike that in *Limulus*, where both —S—S— bonding and aromatic tanning occur (Lafon, 1943*a*). Further support of the above assumption is obtained by treating sections of the scorpion cuticle with an alkaline solution of sodium sulphide, which affects the —S—S— bonds and renders the protein soluble (Brown, 1950). Pieces of hard cuticle of the scorpion after treatment with sodium sulphide become swollen and soft; and when this material is sectioned by the paraffin method, the cuticle appears to be disrupted. The epicuticle at various regions was separated from the exocuticle, which lost markedly its amber coloration. Both exocuticle and the epicuticle now appeared more susceptible to the action of mineral acids. When treated with hot concentrated nitric acid, the exocuticle dissolved and the epicuticle completely broke up leaving oily globules, whereas in normal sections of the cuticle the epicuticle was unaffected and the exocuticle resisted for some time the action of nitric acid. Obviously the effect of sodium sulphide is to bring about a loss of resistant properties. Another effect of sodium sulphide treatment is an assumption of reactive properties to stains. The tanned exocuticle in the normal cuticle is unresponsive to iron haematoxylin, but after treatment with sodium sulphide, it takes up a deep blue or black colour. With Mallory it stains a dark reddish colour which is different from the staining of the untreated cuticle. The significance of the effect of sodium sulphide on the reactivity of the proteins to stains is not clear. The results suggest a close parallel to a similar resumption of staining property by the tanned epicuticle of insects after treatment with diaphanol, which breaks up the aromatic nuclei in a tanned protein. With the knowledge of the chemical reaction of sodium sulphide on —S—S— bonds it appears reasonable to infer that the bisulphide linkages may be closely associated with a tanning process in the exocuticle.

Epicuticle

The existence of an epicuticle in arachnids and myriapods has been a matter of controversy. While authors like Langner (1937), Fuhrmann (1921), Lees

(1946, 1947) affirmed its presence, Browning (1942) does not refer to an epicuticle in *Tegenaria* and Blower (1951) considered that an epicuticle does not exist in Myriapoda. In *Palamneus* the optical appearance of the cuticle is very similar to that of myriapods and it was therefore of interest to examine whether, as suggested by Blower, the thin colourless layer external to the exocuticle is an optical phenomenon or gives evidence of a layer corresponding to the epicuticle of insects and Crustacea.

In support of the view that the colourless layer is not merely an optical effect, it is observed that this layer may be separated by treatment of the entire cuticle with concentrated potash at 180° C. for several hours and sectioning it by the paraffin method. Such sections revealed a thin colourless layer, separated from the exocuticle but at various points still retaining its position in contact with the exocuticle, and recalling strongly the appearance met with in normal cuticles. The integrity of the layer is further borne out by treatment with chlorated nitric acid. The endocuticle dissolves rapidly and on heating the entire tanned part also disappears, leaving a thin colourless membrane corresponding to the outermost layer which resists the action of the acid for several days. Cloudsley-Thompson (1950*a*), by a similar treatment with chlorated nitric acid, demonstrated the presence of a colourless epicuticle in a number of arachnids. From the above observations it appears that there can be no doubt that the colourless layer seen in sections is a constituent part of the cuticle and corresponds to the epicuticle. There is some evidence that the epicuticle is bounded externally by a thin membrane which stains blue with Mallory and haematoxylin in a hardened cuticle. The membrane is not easily distinguishable in the soft regions such as the cuticle of the arthrodial membrane. However, this affords suggestive evidence of a two-layered epicuticle similar to that of Crustacea (Dennell, 1947*b*; Krishnan, 1951).

From chemical tests (Table 1) it is seen that the epicuticle is resistant to the action of concentrated mineral acids and also alkalis. The negative reaction obtained with the chitosan test may show that it is non-chitinous. The presence of reducing substances are indicated by a positive reaction with Fehling's solution. Although the nature of the reducing substances indicated is not clear, a feeble effect seen with ferric chloride and ammonia may be suggestive of their phenolic nature. Prolonged treatment with ammoniacal silver nitrate gives a brown coloration in the epicuticle though less intense than that in the exocuticle, which according to Lison (1936) may be interpreted as showing the occurrence of polyphenols. A prominent chemical constituent of this layer appears to be lipoid, very distinctly revealed by staining with sudan IV. There is evidence that lipoid is indicated in the dermal gland ducts opening into the epicuticle as seen by a positive reaction with sudan IV. Similar conditions have been noted in myriapods (Langner, 1937; Blower, 1951). The occurrence of lipoid in the epicuticle has been widely recorded in other arthropods also. In insects the importance of lipoids of the cuticle in the water economy of the animal and the permeability of the cuticle has received considerable attention.

t appears possible that a similar role may be played by the lipoids of the picuticle in the scorpion also.

In a tanned and hardened cuticle the epicuticle is unresponsive to stains; ut in the soft regions such as the arthrodial membranes where the epicuticle s flat and thrown into folds, it stains a deep blue with haematoxylin and red ith Mallory. The condition of the epicuticle in this region recalls what has een described by Lees (1946) in the soft and extensible regions posterior to he scutum in *Ixodes*. Apparently as a result of the assumption of hardness the picuticle loses its reactive property to stains. However, treatment with sodium ulphide seems to restore the staining property, for when pieces of cuticle are reated with sodium sulphide, the epicuticle takes up a blue and red colour in ections stained with haematoxylin and Mallory respectively. It has already een observed that the exocuticle behaves similarly after sodium sulphide reatment and it has been suggested that the resumption of staining property ay result from the breaking up of the —S—S—bonds, in correspondence with he effect produced by diaphanol on a tanned cuticle. The significance of this ssumption, if valid, would be to indicate the occurrence of —S—S— bond- ng in the process of hardening of the epicuticle. The presence of organic ulphur revealed by the positive lead acetate reaction, and the swelling effect roduced by thioglycollate solution, lend further support to the view that the picuticle though formed of proteins (as in insects) differs from the latter in he presence of —S—S— groups, which are probably involved in a process of ardening similar to the phenomenon of keratinization.

iscussion

The morphological features of the scorpion cuticle present resemblances o those of the cuticle of other arthropods in a number of features. In the resence of two fundamental layers—an epicuticle and endocuticle—and in e formation of an exocuticle as a result of changes taking place in the outer yers of the endocuticle, it conforms to the type met with in insects and rustacea. The scorpion cuticle in its optical appearance and staining re- ctions appears to approximate to the condition seen in myriapods, with hich it seems to agree closely, although Blower (1951) considered that an picuticle is wanting in the latter group. From the evidence presented in the oregoing pages there appears little doubt that the colourless outermost layer f the scorpion cuticle is really the epicuticle. This observation is in accord ith what has been described in other arachnids. Lees (1946, 1947) noted that ixodid ticks there is a well-developed epicuticle showing the chemical haracteristics of the insect epicuticle, and as in the latter, formed of a basal yer of cuticulin external to which are the polyphenol and wax layers. In *rnithodorus moubata* there is in addition a cement layer covering the wax, he general pattern being identical with that in insects like *Rhodnius* (Wiggles- orth, 1947). Although it cannot be said that in scorpions such a close parallel- sm could be established, the presence of an epicuticle giving evidence of the ccurrence of proteins, polyphenols, and lipoids is not inconsistent with the assumption that it conforms to the type met with in insects and Crustace But a noteworthy chemical feature of the scorpion epicuticle and one in whic it differs markedly from its counterparts in insects is its resistance to th action of hot alkalis. In the arachnid *Ixodes*, Lees (1947) showed that th epicuticle is dissolved in hot caustic potash. This feature is found in all insect Obviously the basal layer of the epicuticle of the scorpion is different i chemical composition from the cuticulin of insects. That this difference attributable to the presence of cystine sulphur and the formation of disulphi bonding in the epicuticle appears probable.

From a study of the scorpion cuticle it is clear that the amber-coloure layer of the cuticle is the exocuticle in spite of its apparent resemblance to th epicuticle of insects (especially in its staining reaction). Blower (1951) dre attention to a similar resemblance with reference to the myriapod cuticl However, in view of the presence of chitin in this layer and its structura features there can be no doubt as to its homology. Browning (1942) describe a similar amber-coloured exocuticle in *Tegenaria*. Very little is known of th condition of the exocuticle in other arachnids.

The endocuticle in its structural features agrees with the condition described in insects. These similarities have been emphasized by Lees (1946) i *Ixodes* and Sewell (1951) in *Tegenaria* among the arachnids. But the chemica features differ from those of the insect endocuticle and in these respect approximate to the myriapod condition (Blower, 1951). It has been pointe out by Blower that the protein impregnating the chitinous matrix of th cuticle is unlike that in the insect cuticle, in possessing resistant propertie and during hardening of the cuticle undergoes changes resulting in th acquisition of greater resistance, a development of amber colour, and finall a loss of staining reaction. Similar conditions prevail in the scorpion cuticle in which the entire endocuticle is impregnated with a protein, which possesse resistant properties and which in the outer regions gets sclerotized to form a amber-coloured exocuticle. From chemical tests it is clear that the protein o the scorpion cuticle may not be identical with that described in insects (Trim 1941; Dennell, 1946; Fraenkel and Rudall, 1947). Further, there is som evidence to suggest that the mechanism of hardening of the exocuticle ma also be different from what has been observed in insects (Pryor, 1940*b* Dennell, 1947*a*). Although the occurrence of phenolic substances and th changes resulting from a treatment with diaphanol indicate tanning of thi region, the presence of organic sulphur is at variance with what is known o the insect cuticle. It has been pointed out that in insects such as *Ephestia* an *Calliphora* the cuticle does not contain any sulphur nor is it softened by treatment with alkaline sodium sulphide (Pryor, 1940*a*). In view of the result obtained above, it is reasonable to assume the possibility of —S—S— group forming linkages in the hardening of the cuticle. That such a condition is no uncommon in other arachnids is shown by the occurrence of Quinone tannin and —S—S— bonding in the cuticle of *Limulus* (Lafon, 1943*a*) in which high sulphur content has been reported. On account of such features th keletal proteins of the scorpion and *Limulus* may be said to be of a similar ype and different from those of the insects and Crustacea. It has been ob-erved that the scleroproteins of *Limulus* resemble the keratin of vertebrates, ut Brown (1950) pointed out that the mere presence of —S—S—linkages is ot sufficient to justify its assignment to keratins unless it also conforms to a eratin type of X-ray diffraction; for there are biological proteins containing —S—S— bonds but not showing the type of X-ray diffraction characteristic f keratin. The significance, if any, of the occurrence and role of sulphur in he cuticular proteins of *Limulus* and scorpion is not obvious, although a vider study of the arthropod cuticle from this point of view may be of great nterest.

This work was carried out at the University Zoological Research Labora-ory, Madras. I am thankful to the Syndicate of the University of Madras for enerous facilities given me. I am indebted to Prof. R. Dennell of the Uni-ersity of Manchester for help and advice in the course of this study.

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The Origin of Proteid Yolk in *Drosophila melanogaster*

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SUMMARY

1. The origin of the proteid yolk granules in the eggs of *Drosophila melanogaster* has been traced to precursors embedded in the matrix material of flakes which are emitted by the nucleoli of the nurse cells.

2. These emissions are of a lipoidal nature, as shown by their behaviour towards fixatives and stains. They have never been observed to come from the nucleolus of the germinal vesicle.

3. The precursors, once released from the matrix of the flakes, develop directly into individual proteid yolk granules.

INTRODUCTION

IN a previous paper (1952), I reported evidence tracing the development of the proteid yolk granules in *Drosophila* eggs to what I described as the yolk flakes sent into the oocyte by its nurse cells. Such yolk granules were not observed to arise in connexion with any of the cytoplasmic elements. Each flake consists of a matrix material in which are embedded minute and intensely staining granules. It is these minute granules, the so-called yolk precursors, which, once transported into the oocyte, develop into individual proteid yolk granules (fig. 1A). Owing to certain peculiarities of the yolk flakes, I was, up to the time of my previous report, not sure of their origin. I was unable to say whether these flakes had a nuclear origin or arose independently in the ground cytoplasm of the nurse cells. Since then, I have obtained slides more favourable for study. The observations made on these slides warrant a short communication on this particular aspect of vitellogenesis in *Drosophila.*

MATERIAL AND METHODS

For the source and method of culture of the flies used, readers are referred to my previous paper (1952). Champy-Benda slides have again been much relied upon for observation because such slides, if well differentiated, demonstrate very clearly the flakes and their origin. Confirmatory evidence has also been obtained, however, from material fixed in Bouin, Helly, La Cour, Gatenby, Sanfelice, and mercuric chloride acetic acid, and stained in Mallory's triple method as modified by Krichesky, iron haematoxylin eosin, and Mann's methyl blue eosin. Feulgen and Unna's pyronine/methyl green tests, made largely with other problems in view, have also yielded valuable information.

[Quarterly Journal of Microscopical Science, Vol. 94, part 1, pp. 23-28, Mar. 1953.]

OBSERVATIONS

Very early in the development of a cyst, when the oocyte has just become distinguishable from its nurses, yolk flakes can be seen in the cytoplasm of both the oocyte and its nurses (fig. 1B). The flakes at this time are very small, irregular in shape, and, unlike those in older cells, they stain intensely and homogeneously. They are so small that it is difficult to tell whether or not they are 'flakes'. Were there no intermediate stages connecting these very early flakes with the later easily recognizable ones, showing well differentiated dark yolk precursors embedded in a more lightly coloured matrix, it would be very difficult not to regard them as different categories of structures (fig. 1 D–G). In fact, I was inclined for a time to regard the early flakes as something similar to what Gresson (1931) reported in the young oocytes of *Periplaneta*, in which they were observed to originate as nucleolar emissions, to pass out into the cytoplasm and eventually to disappear, without appearing to be directly concerned with proteid yolk formation. The small flakes in the young oocyte of *Drosophila*, however, do not disappear in the cytoplasm, but have been observed to be directly concerned in proteid yolk formation. They are transported from the nurses into the oocyte in the mitochondrial stream and remain with most of the mitochondria within an area which eventually will be occupied by what was called the 'yolk nucleus' in my previous report (fig. 1C). There they release the minute yolk precursors which in time develop into individual proteid yolk granules. That the small flakes show no differentiation in their structure but stain homogeneously must be due to the close packing of the intensely staining yolk precursors in a relatively small mass of matrix material.

As to the origin of the flakes in the nurse cells, I have now evidence that they have a nucleolar origin. In Champy-Benda slides the chromosomes in very young nurses stain a light purple, the nucleolus yellowish, and the flakes intense purple. The flakes are frequently seen in contact with the nucleolus or lying free in the nuclear sap, but never really in contact with the chromosomes which in very young nurses are in several bunches and not in a reticular condition (fig. 1C). Some of the flakes are in contact with the nuclear membrane, both inside and outside it. Owing to the small size of the flakes in very young cells, it is difficult to say if some of them actually pass through the nuclear membrane into the cytoplasm. But in older cells, in which the nucleus begins to show a wrinkled surface and in which the flakes have become bigger, the flakes do appear to be passing bodily through the nuclear membrane into the cytoplasm (fig. 1 E–G).

Elsewhere in this paper, it has been mentioned that there are intermediate stages between the condition as shown by the small flakes in very young nurses and that shown by the bigger flakes in older cells. In this connexion, it is interesting to notice that as the nurse cells grow larger, their nucleolar mass also increases in volume, and the flakes which the nucleolus throws off also become larger (fig. 1 D–G). Further, it seems clear that more of the flakes which are still in contact with the nucleolus are homogeneously stained and comparatively smaller than those lying free in the nuclear sap. And, if one compares the average size of the flakes outside the nucleus with those within it, it will be found that those in the cytoplasm are on the whole bigger. This state of affairs is well illustrated by figure 1 C–G. It would seem that once a flake is free from a nucleolus, it begins to unfold or swell in some way. The ultimate size which a flake may reach before the final disintegration of its matrix when it is in the oocyte seems to be determined by the original mass each flake receives from the nucleolus. The final dissolution of the matrix of a flake in the oocyte may perhaps represent the end of a process which begins almost as soon as the flake is freed from the nucleolus.

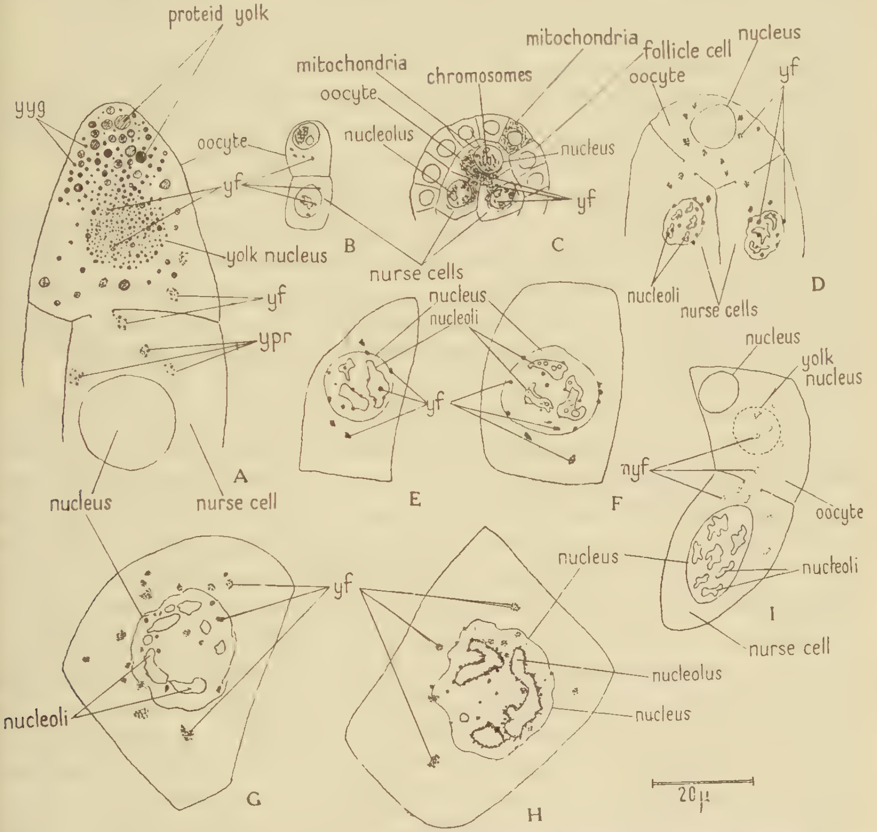

FIG. 1. A, oocyte and portions of nurse cells; B, oocyte and nurse cell; C, tip of a cyst; D, oocyte and portions of nurse cells; E–H, nurse cells; I, oocyte and nurse cell. All except I are Champy-Benda preparations; I is a mercuric chloride/acetic preparation stained in methyl blue/eosin. *nyf*, negative image of yolk flake; *yf*, yolk flake; *ypr*, yolk precursor; *yyg*, young proteid yolk granules.

The nucleoli in the nurse cells are not differentiated into oxyphil and basiphil portions at any stage of development. In Champy slides, an overwhelming majority of them appear homogeneous, though in material fixed in fluids containing acetic acid vacuoles appear in them. But these vacuoles do not show any different staining behaviour from the nucleolar material itself. It certainly cannot be said that it is the vacuoles that become the flakes, as reported by Gresson in *Periplaneta* (1931). Relying largely on morphological observations, I am inclined to believe that the flakes are the result of localized exudation by the nucleolus at specific spots on its surface. I have also seen cases in Champy-Benda material wherein the whole surface of the nucleolus appears to be coated with a thin layer of material having the same staining reaction as the flakes (fig. 1H). Such a condition has been too often encountered to be ignored. It is, therefore, also possible that the yolk flakes arise as a result of localized concentrations of a material given off by the nucleolar mass all over its surface. At any rate, the flakes are not just broken bits of nucleolus; although at a stage of development, as shown in fig. 11, the nucleolus does appear broken into very many little bits; but these are much smaller and more numerous than they are shown to be in the figure. But I have never seen any indication that the little bits are capable of transforming bodily into yolk flakes.

It seems that the yolk flakes are of a lipoidal constitution. They are best preserved and demonstrated in Champy-Benda slides in which the material undergoes a prolonged soaking in potassium dichromate. They have also been demonstrated, though less well, by both iron haematoxylin and Benda stains after Gatenby fixation. In material treated in acid fixatives they are not observed. In mercuric chloride acetic material stained in methyl blue eosin, however, I have repeatedly seen their negative images in the cytoplasm (fig. 11). In such slides, the nuclear sap appears colourless, and that, I believe, accounts for the apparent absence of negative images within the nucleus. This same reason may also account for the difficulty in detecting negative images of the flakes in nuclei fixed in Bouin and coloured by Mallory's triple stain as modified by Krichesky, and also in Sanfelice material stained in iron haematoxylin. The cytoplasm of the cells fixed in these fixatives are quite rich in vacuoles. Though some of the vacuoles would match the shape and size of the flakes, it is not safe to regard them as their negative images.

The Feulgen test applied to material fixed in Champy, Helly, Sanfelice, La Cour, Bouin, and mercuric chloride acetic all have given positive results only to chromosomes: nothing else in the nurse cells has given positive reaction to this test. Unna's stain for ribonucleic acid has also been applied to Champy and Helly slides, both before and after digestion with ribonuclease. Repeated tests have convinced me that the flakes do not contain any ribonucleic acid. At least, if there is any ribonucleic acid in the flakes, it is not present in an amount demonstrable by Unna's stain. The same test, however, has repeatedly demonstrated the acid in the cytoplasm and the nucleolus.

DISCUSSION

Although there does not seem to be a general agreement on the nucleolar origin of the proteid yolk in the insect eggs, there are, nevertheless, a number of reports supporting such an origin. For instance, Hogben (1920), Nath and Mohan (1929), and Gresson (1931) have reported it in *Periplaneta*; Bhandari and Nath (1930), in *Dysdercus*; Nath and Mehta (1929), in *Luciola*; and Gresson (1929), in Tenthridinidae. Evidently, so far as the origin of the proteid yolk is concerned, my observations only add to the list another form of insect. There are, however, at least three characteristics in *Drosophila* which distinguish it in this respect from the other insects thus far reported: (1) that the nucleolar emissions are not demonstrated in acid fixatives: their behaviour to fixatives and stains indicate that they are of a lipoidal constitution; (2) the presence of yolk precursors in the nucleolar emissions; and (3) that such emissions are contributed exclusively by the nurse-cell nucleolus: the nucleolus in the germinal vesicle has never been seen to produce emission.

I cannot recall that any lipoidal emissions having to do with the production of proteid yolk in any animal have ever been reported. The case reported by Subramaniam and Aiyar (1935) in *Acentrogobius* is different from that of *Drosophila*, since there the nucleoli give unmistakable fat reaction to Scharlach R and sudan III. Besides, evidence was cited by the authors to support their view that fat and not proteid yolk arises in the oocytes by passage of material through the nuclear membrane. In this connexion it may not be amiss to recall that Mensinkai (1939) has succeeded in colouring the nucleoli in the onion root-tips orange-red with sudan III and black with 1 per cent. osmium tetroxide. Although I have not tried the two reagents on fresh material of *Drosophila*, it is nevertheless a fact that I have never detected any flakes in unstained and unbleached Champy slides.

The lipoidal nature of the emissions, leading to their non-appearance in acid-fixed material, and their ability to expand after being freed, thus changing their size and appearance, contributed greatly to my reluctance to come to a definite conclusion, in my previous report, as to the precise site of origin of the yolk flakes.

The presence of the proteid yolk precursors seen in *Drosophila* seems unique among insects. This uniqueness, however, may be more apparent than real. Gresson (1931) revealed, in the second type of nucleolar emissions of the *Periplaneta* oocyte, small dark granules within a clear substance. These may very well be homologized with the yolk precursors in *Drosophila*, although Gresson did not report direct observational evidence that such granules transform into the albuminous yolk-spheres. He did say, however, that the second type of nucleolar extrusions 'become vacuolated and undergo fragmentation to form small dark granules which are dissolved, their substance forming clear vesicles which increase greatly in size and form the albuminous yolk-globules'. It seems, then, that both Gresson and I have encountered proteid yolk precursors emitted by the nucleolus, though in *Periplaneta* their transformation into proteid yolk is less direct than in *Drosophila*.

So far as I have studied the germinal vesicle, there is no evidence that its nucleolus exhibits emission. All the yolk flakes are sent into the oocyte as a result of emissions on the part of the nurse-cell nucleolus. This does not mean, however, that the germinal vesicle remains quiescent during the period of vitellogenesis in *Drosophila*. Many interesting changes have been seen in it during this stage of development. But since observations are still going on, I hope to report the information concerning the germinal vesicle in a future communication.

This work is a part of a project supported by the Washington State Initiative 171 Research Fund for Biology and Medicine.

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The Preservation and Investigation of Plant Mitochondria

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SUMMARY

A method for making squash preparations of plant root tip cells is described. This entails the use of an inert preserving substance, polyvinyl alcohol. The advantages of the method are the preservation of the cytoplasmic bodies, including the mitochondria; the retention of fat droplets inside the cells; the fact that the nucleus remains optically homogeneous; and the fixing of free aldehyde groups and of diffusible substances in the cytoplasm. It is a relatively rapid and simple technique, and the cells produced by it resemble closely those produced by the method for making living squash preparations of such cells.

INTRODUCTION

METHODS of fixing plant cells in such a manner as to preserve the cytoplasmic components, in particular the mitochondria, are few and complicated. This is due to the nature of the mitochondria, which are destroyed by acids, by dichromate mixtures of more acid pH than about 4·2–5·2 (Zirkle, 1929), and by alcohol and other fat solvents. Squash preparations are usually produced by acid hydrolysis of the intercellular cement, e.g. as in the standard aceto-carmine method and when the Feulgen hydrolysis (6 minutes in N.HCl at 60° C.) is used to separate the cells. Such methods destroy plant mitochondria. The tissue must therefore be fixed in such a way that the mitochondria are rendered resistant to fat solvents and then embedded and sectioned. Probably the Newcomer-Zirkle technique (see, for example, Newcomer, 1940) based on the work of Zirkle on dichromate fixatives (Zirkle, 1929; 1931) is one of the most useful. The roots may be prefixed to improve nuclear fixation, but in our own studies this step has been omitted and the roots are placed for 24 hours in a chromium fixative at the correct pH (1·25 g. potassium dichromate, 1·25 g. ammonium dichromate, 1·0 g. copper sulphate, made up to 100 ml. with distilled water). They are then washed in running tap water for 24 hours, after which they are immersed in 2 per cent. osmium tetroxide for 6 days and again washed in running water for 24 hours; they are then embedded by standard techniques. The sections are bleached in 1 per cent. potassium permanganate for 5 minutes and then treated with 3 per cent. oxalic acid either for 3 minutes or until they are decolorized. This method, however, has many drawbacks. It is lengthy, and fixation of root tips of the broad bean, *Vicia faba*, is variable and patchy, while results obtained with onion root tips have been unsatisfactory; the technique was originally designed

[Quarterly Journal of Microscopical Science, Vol. 94, part 1, pp. 29-35, Mar. 1953.]

for maize roots. The enzymatic digestion of the intercellular cement (for references see Chayen, 1949; see also Chayen, Davies, and Miles, in press) is rendered more difficult by this method, so that the cells cannot be studied easily in squash preparations. Moreover, it interferes with the fluorescence of the cells, perhaps partly by washing fluorescent material out of the cytoplasm and partly by its mode of fixation, which makes them useless for primary fluorescence microscopy; it also affects the ultraviolet absorption characteristics of the tissue, probably because of the presence of chromium in the sections.

Freezing-drying of root tips was attempted for us by Dr. L. G. E. Bell, but only very occasionally were satisfactory results obtained. When successful, excellent preservation of cytoplasmic detail was observed, but in most of the roots all such details were obliterated. This was believed to be due either to bad impregnation with wax after the freezing and drying, or to premature warming of the specimens before drying, owing to the small bulk of the root tips (Bell, unpublished).

Thus, it was necessary to devise a new technique. Mitochondria are so easily destroyed that it seemed as if the ideal fixative would be some inert substance that could mummify the cells. It had previously been discovered (Chayen, unpublished) that polyvinyl alcohol (PVA) preserves insect blood cells, which normally disintegrate within 15 minutes of being placed on a slide. It was therefore decided to test whether this substance would also preserve the delicate mitochondria of plants. Polyvinyl alcohol had been used by Downs (1943) as a medium for mounting and clearing biological specimens and for slowing the movements of living mosquito larvae and motile microorganisms, and by Lubkin and Carsten (1942) as an embedding agent for microtome sectioning.

In the present method, roots or root tips are placed for 1 day in an approximately 5 per cent. aqueous solution of polyvinyl alcohol. They are then transferred to an aqueous solution of a pectinase preparation to remove the inter-cellular cement. After a suitable period (one to several hours depending on the tissue and the enzyme solution) the tissue can be macerated very readily by gently tapping it with a needle. At this stage stain may be added, such as a very dilute aqueous solution of Janus green, and the cells are then mounted in a more viscous polyvinyl alcohol solution which has been plasticized with glycerine to prevent cracking of the PVA on drying. A more complex mounting agent, containing PVA as the major constituent, has been described by Gray and Wess (1950); it has the advantage that it sets to a cement having a constant and known refractive index.

SOLUTIONS

I. *For fixation*

1. *Stock solution.* Polyvinyl alcohol (PVA) powder is troublesome to dissolve. It was more convenient to prepare a stock solution from which samples could readily be diluted as required. To make this stock solution 30 g. of PVA powder (obtained from Vinyl Products Limited) are mixed carefully

with sufficient glass-distilled water to form a stiff paste. This is best done in a large pestle and mortar in order to break up any lumps. More water is then added to bring the final volume of water in the solution to 100 ml. and is worked well into the paste. This solution is heated on a water bath, the temperature of which reaches about 90° C., until it is completely cleared of foam; this process usually takes about 30 minutes. Since the cleared solution usually sets to a weak jelly after a few days, it is stored in a wide-necked jar with ground-glass stopper, thus reducing further evaporation and facilitating the removal of samples of the jelly.

2. *Preserving* (*fixing*) *solution*. Five g.of the jellied stock solution are added to 20 g. of glass-distilled water and warmed until all the jelly has dissolved. This gives an approximately 5 per cent. PVA solution in water. Unfortunately micro-organisms grow in this solution so that it has to be made up freshly from the stock solution just before it is to be used. Some roots are especially liable to attack by micro-organisms and it has been found that better preservation of such roots is obtained in a mixture of 4 volumes of approximately 5 per cent. PVA to 1 volume of absolute ethanol. A milky appearance, noticeable at the meniscus when this mixture is prepared, disappears on shaking.

II. *For mounting preparations*

An aqueous solution of PVA is prepared by dissolving 4 g. of PVA powder in 20 ml. of water in a manner similar to that described for preparing the stock preserving solution. To the cooling solution is added 20 per cent. (by weight) of glycerol. The mixture is well stirred and is ready for use when cool. It may be left in a jar, and keeps for two or three days. Lubkin and Carsten (1942) give a similar formula for an embedding medium.

III. *Pectinase preparation*

The pectinase is extracted, as required, from samples of *Penicillium digitatum* which has been grown on bran and subsequently dried (see Chayen, 1949). The samples used in the present studies were different from those in previous investigations (e.g. Chayen, Davies, and Miles, in press; Chayen 1949) and gave suitable maceration in a very much shorter time. The enzyme is extracted by shaking 1 volume of bran with 4 volumes of glass-distilled water and leaving the suspension to stand for 1 hour. It is then centrifuged and the supernatant, diluted twice, is used. This extract appears to be roughly equivalent to a 5 per cent. solution of the pectinase powder sold by Nutritional Biochemicals Corporation (Cleveland, Ohio), a sample of which was kindly given us by Dr. R. Brown of Leeds.

METHODS

1. *Squash preparations of onion root-tip cells*

The pectinase technique for living root-tip cells described by Chayen (1949) could not be used to separate the living meristematic cells of onion roots. When so treated, the onion roots degenerated and produced offensiv odours. The apical 4–5 mm. of onion roots were therefore placed in th PVA-ethanol preserving mixture for 24 hours. For convenience, and to exclude the growth of micro-organisms, this was done in two stages: roots wer placed in the solution at 11 a.m. and transferred to a fresh, similar sample a about 5.30 p.m. It was desirable to use about 15 ml. of preserving solution t 10 root tips. After a total of 24 hours in the preservative they were transferred to the pectinase solution for $1\frac{1}{2}$ hours, after which the meristem cell separated very readily when squashed in water with a broad-bladed scalpe or with a spear-shaped needle.

When PVA-glycerol was used as the mountant, the cells were macerated in a small drop of water and stained, if required, with a very dilute, aqueou solution of Janus green; the mountant was dropped on to the cells, allowed to mix with the water, and the coverslip was then placed in position.

2. *Squash preparations of bean root-tip cells*

The apical 3–4 mm. of lateral roots of *Vicia faba* were immersed in the approximately 5 per cent. PVA solution for 24 hours and were then treated with the pectinase solution. Satisfactory maceration was only obtained after about 8 hours' treatment. When living lateral root tips were treated with a similar concentration of pectinase to produce living squash preparations adequate separation of the cells was obtained only after a similar period (i.e about 8 hours), although bean root tips which were fixed in acetic-alcohol required treatment for only about 1 hour.

Bean root tips were also well preserved by fixation in a mixture of 9 volumes of the approximately 5 per cent. PVA solution to 1 volume of absolute ethanol.

RESULTS

Cells of onion root tips prepared by this technique were very different from similar cells prepared by such methods as the aceto-carmine squash method, acetic alcohol fixation followed by Feulgen hydrolysis, and fixation in acetic-alcohol followed by maceration by a pectinase. The cytoplasm of cells prepared by these three techniques was not homogeneous; it showed coarse precipitation of the cytoplasmic protein by the fixative, but was free from large granules and similar separate bodies. When cells which had been fixed with acetic acid, alcohol, or a mixture of the two, were treated, without hydrolysis, with the Feulgen reagent (as is frequently done as a control for true Feulgen staining), no colour was developed. On the other hand, the cytoplasm of meristematic cells of onion roots prepared by the PVA method appeared homogeneous but contained granules of various sizes, large droplets probably of lipoidal matter, and, when tested without hydrolysis with the Feulgen reagent, it stained pink, the colour being mainly associated with the mitochondria-like granules. It is probable that the colour is due to free aldehyde or similar groups present in lipoidal material. Such material is lost from the cells on treatment with fat solvents and so this reaction is not observed in tissues fixed with acetic acid and alcohol.

A very dilute aqueous solution of Janus green was used to differentiate the mitochondria from droplets and other bodies. The appearance of the cytoplasmic bodies and the general form of the cells resembled fairly closely what had been observed in living squash preparations and in the best frozen-dried material. When, however, a root which had been fixed with PVA was heated in normal hydrochloric acid for 6 minutes at 60° C., stained with the Feulgen reagent, macerated and examined directly (or made permanent by mounting in PVA-glycerine), it was found that the cells resembled those of roots fixed in, for example, acetic-alcohol, and stained by the Feulgen reaction. Whereas the unhydrolysed nuclei prepared by the PVA method were optically homogeneous structures containing nucleoli, after the Feulgen hydrolysis and staining they were granular and well stained, with the nucleoli and cytoplasm colourless. No discrete bodies were observed in the cytoplasm of meristematic cells after such treatment.

In studies of living squash preparations of bean root tips it was found that some ultraviolet-absorbing material was lost from the cytoplasm during the course of preparation. Such cells also emitted little primary fluorescence (see, for example, Chayen, 1952). PVA-fixed cells, however, showed intense primary fluorescence both in the cytoplasm and in the granules which surrounded the nuclei. Nucleoli also fluoresced. The fluorescence was not due to the presence of PVA, which does not emit visible light when excited at the wavelength used in this study, namely 3,650 A. Fluorescence was not observed in frozen-dried material.

DISCUSSION

The polyvinyl alcohol method, used in conjunction with enzymatic degradation of the intercellular cement, provides a very simple and fairly rapid method of preparing plant cells for cytological, as opposed to purely cytogenetical, examination. Such preparations are superior to those produced by most standard cytological techniques in that the cytoplasmic particles and droplets are well preserved; there are no signs of coagulation or crude precipitation of cell proteins; the nucleus remains optically homogeneous with the nucleic acids apparently in the disperse phase (see Ris and Mirsky, 1949). Furthermore, not only are fat droplets when present, as in the onion, well-preserved, but free aldehyde and similar groups probably associated with lipoidal material, which are normally lost on fixation, have been demonstrated by the recoloration of Schiff's reagent (the Feulgen reagent) without prior hydrolysis (see Danielli, 1949; Cain, 1949). The method is so easy and so reproducible that it has been used successfully by an undergraduate class. An additional advantage is that soluble substances diffusely distributed in the cytoplasm do not appear to be lost from the cells.

On the other hand, if such cells are treated with the reagents used in the production of standard cytological preparations, all the cytoplasmic organization and optical homogeneity are lost and the cells resemble those prepared by standard techniques alone.

This raises the question of the mode of action of the PVA. Baker (1945) defines fixatives as 'solutions that prevent decay and autolysis, render the substances of the cell insoluble, and lessen its subsequent shrinkage and distortion'. He considers one of their functions is to prevent subsequent treatment from causing unwanted changes. In this respect PVA is not acting as a true fixative in that the structures preserved will not withstand further treatment with, for example, xylene in the dehydrating, clearing and mounting schedule ordinarily used. It would appear to act more as a preservative or possibly, as a narcotic. This might have been its action when used by Downs (1943) to stop the movement of micro-organisms and of mosquito larvae; if they were exposed to PVA for short periods and were then washed thoroughly they recovered and behaved normally. If the treatment, especially with the micro-organisms, was prolonged, however, death ensued. It may be that root-tip cells react in a similar way.

Brown (1951), using a pectinase method for isolating root-tip nuclei, found that they broke down unless some long molecule such as pectin or peptone was added. Such molecules appear to influence active surface groups and so stabilize the system. It is possible that short treatment with PVA has a similar effect on the whole cell, apparently inhibiting active surface groups and so retaining cytoplasmic material which may be lost even from cells produced by the method for living squash preparations. It may be, however, that it acts by entering the cytoplasm; since it is an inert substance it would thus 'mummify' the cell.

ACKNOWLEDGEMENTS

We wish to thank Professor J. T. Randall, F.R.S., for his encouragement and interest in the whole of the research of which this forms a part. We are also very grateful to Dr. Honor B. Fell, F.R.S., for her guidance and advice. Our gratitude is also due to Professor J. F. Danielli for the stimulating interest he has taken in the problem. The work has been aided by a grant made by the British Empire Cancer Campaign to one of us (J. C.) who wishes to record his gratitude to this organization.

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Some Observations on Mouse Spermatids

By L. G. E. BELL

(*From the Zoology Department, University of London King's College*)

With one plate (fig. 1)

SUMMARY

The preparation of mouse testis for cytological study by use of the freezing and drying technique is described.

The mitochondria of the middle-piece of mouse spermatids in sections stained with iron haematoxylin are shown to be separate granules arranged in a spiral.

Later the granules appear to coalesce to form a continuous structure.

An undulating motion of the middle-piece is identified by exploiting the rapid cooling action of the freezing and drying technique.

MAMMALIAN spermatogenesis has recently been reviewed by Gresson (1951). He emphasized that there is no general agreement as to the nature of the mitochondrial sheath of the middle-piece. The points at issue seem to be whether it is spiral in structure and whether it is granular or a continuous band. Gresson (1950) claimed the sheath is a spiral, workers with the electron microscope (Randall and Friedlaender, 1950; Bretschneider and van Iterson, 1947; Bretschneider, 1949) claimed a spiral structure. This work has been done mainly on mature sperms. Gatenby and Woodger (1921) and Gatenby and Beams (1935) did not support the spiral structure, but gave evidence for the existence of separate granules in the spermatid middle-piece. Benda quoted in Wilson (1925) gave figures showing a granular and spiral structure, though the figures are not clear. While the present paper was being prepared, Austin and Sapsford (1952) published phase-contrast photomicrographs of surviving rat spermatids, and said that the mitochondria form a spiral structure developing from a granular to a continuous spiral. At the present time attention is centred on the cytochemical structure and physiological function of the middle-piece (Rothschild and Cleland, 1952). As a preliminary to cytochemical work, it seemed important to demonstrate the morphology in a convincing way, by using carefully chosen techniques of fixation especially suitable for cytochemistry (Bell, 1952; Gersh, 1952).

METHODS

It was clear that little fresh knowledge was likely to result from the use of chemical fixatives, which have been used many times previously on mouse testis by very competent cytologists. For the work described in this paper, testis tissue was obtained in wax blocks for sectioning by the following technique.

[Quarterly Journal of Microscopical Science, Vol. 94, part 1, pp. 37–40, Mar. 1953.]

The mice were killed by breaking their necks. The testes were quickly removed and small portions were gently spread on very thin celloidin film. Th thickness of the layer was about that of a single tubule. The celloidin was cu into strips and quickly cooled in a bath of propane held near its melting-poi (−190° C.) by immersion in liquid nitrogen (Bell, 1952). The cooled materi was dried *in vacuo* at −40° C. and embedded in paraffin wax (m.p. 54° C. Material prepared in this way could be easily sectioned and most of it showe very good preservation of structure. Sections were cut at 5μ and stained wit iron haematoxylin. The photographs were taken through a Watson 2 mm apochromatic objective, with a Kodak F75 blue filter, on microfile film. Th initial magnification was × 1,200 diameters.

RESULTS

Fig. 1, A shows the middle-pieces of a clump of spermatids. The blac staining mitochondria of the spermatid to the right of the figure are clearl seen to be arranged in a spiral, and to be separate granules approximatel 0·35 μ in diameter. Figs. 1, B and 1, C show cross-sections of such middle-piece at successive levels of focus. That the arrangement of the granules is a spira is shown by the change in orientation of the same middle-pieces in the tw photographs. A model was made by packing glass spheres representing the mitochondria in a cellophane tube with a rubber tube in the centre to represent the axial filament. It was found that when the cross-section appearance wa the same as in figs. 1, B and C, the whole structure had the same relativ dimensions as those shown in figs. 1, A–C. The diameter of the rubber tub necessary to fill the hole through the centre of the model, gave the dimension of the axial filament as approximately 0·15 μ.

More mature spermatids, showing regression of the cytoplasm from the distal end of the middle-piece, gave cross-sections as in fig. 1, D. Here the mitochondria cannot be so easily seen as separate entities and the appearance is that of a cross-section of a continuous spiral. Later spermatids show a much smoother spiral in which individual mitochondria cannot be distinguished.

It was noticed that many of the middle-pieces have an undulating appearance. Tracings were made from photographs of six spermatids found in a single field of a photograph taken with a Leica microscope attachment. It was found that they could be arranged as successive stages in a wave motion starting from the base and moving towards the tip (fig. 2). The type of photograph from which the tracings were made is shown in fig. 1, E–H.

DISCUSSION

The material described in this paper demonstrates that freezing and drying can give preparations of cytological detail. Hoerr (1936), Simpson (1941), and

FIG. 1, A, mouse spermatids stained with iron haematoxylin.
B and C, cross-sections of spermatid middle-pieces at two levels of focus.
D, cross-sections of middle-pieces of spermatids more mature than in B and C.
E–H, mouse spermatids showing undulations of the middle-pieces.

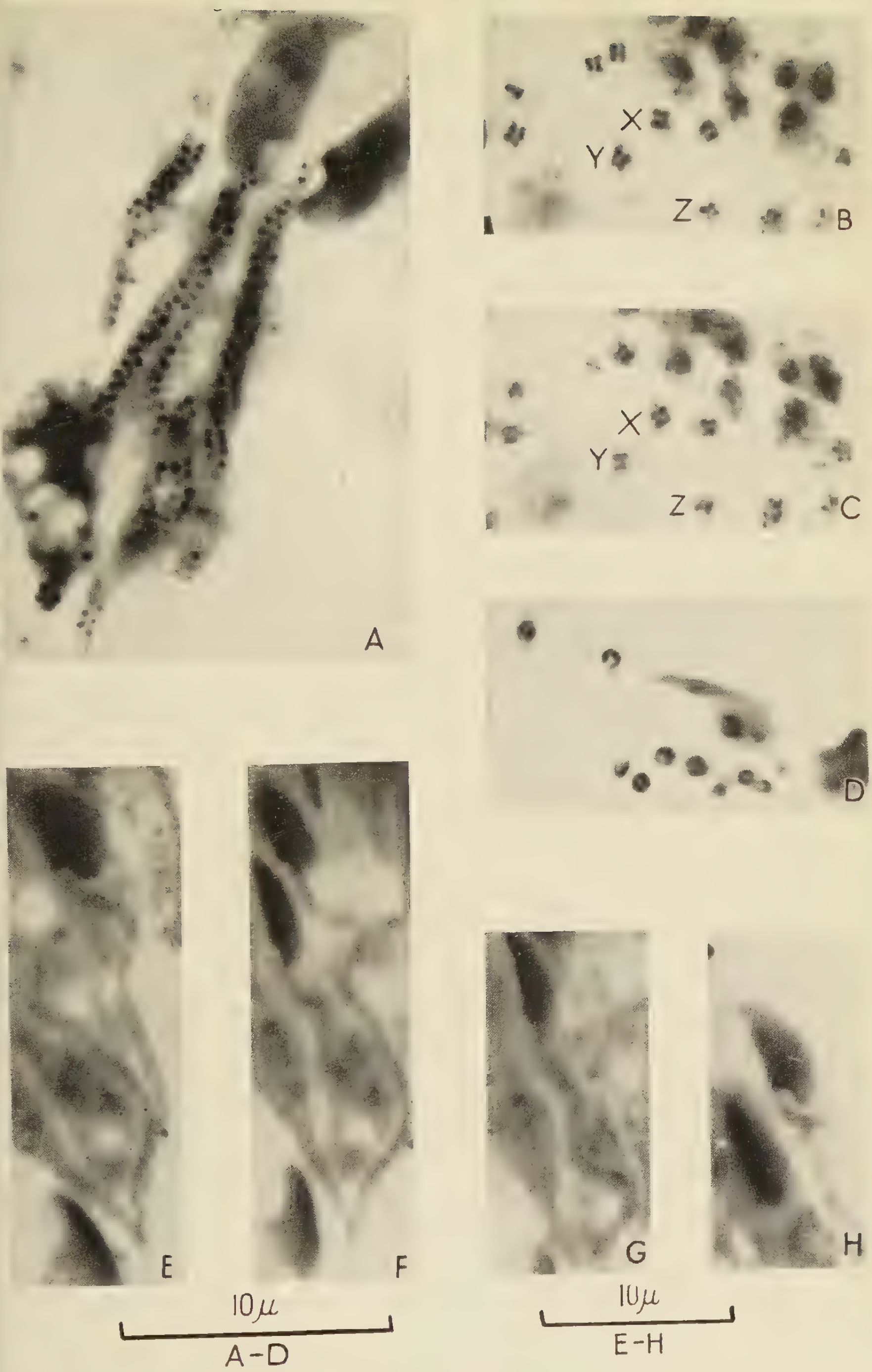

Fig. 1

L. G. E. BELL

Everson Pearse (1951) say that testis is difficult or impossible to preserve by this method. It has been emphasized that the best preservation is obtained by using very thin specimens (Bell, 1952) and the method used here is in effect the rapid cooling of single tubules. It is this which is responsible for the quality of the preservation.

In dealing with structures near the limit of microscopical resolution, it is quite possible for the shrinkage accompanying chemical fixation and dehydration to bring the structures below the limit of resolution. A line of granules such as these middle-piece mitochondria would show as a continuous line in the light microscope if shrinkage had brought them much closer together. Such factors may well explain some of the controversies about such small structures. The shrinkage in freezing and drying is small and may in such material have this extra advantage over the traditional techniques.

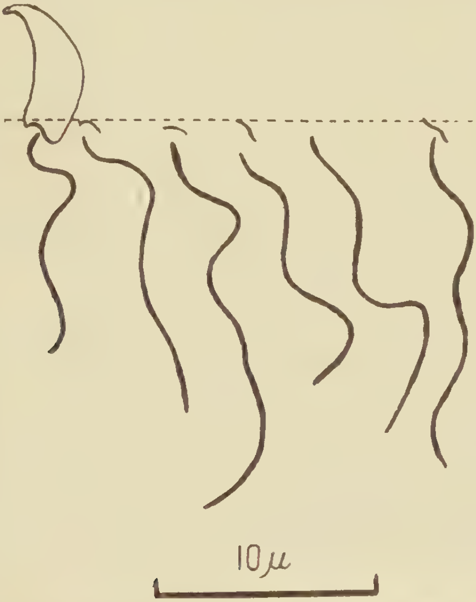

FIG. 2. Tracings of middle-pieces from photographs, arranged to show them as stages in an undulating movement.

The material illustrated by the photographs in this paper gives a clear and consistent picture of the spiral and granular nature of the middle-piece of the mouse spermatid. The forces responsible for this structure are not so clear. Models show that the mitochondria are close-packed in contact. The simplest way of maintaining such a structure is to have a constraining outer sheath. The photographs give no indication of such a structure, but this by no means indicates that it does not exist. It is perhaps a change in the properties of such a sheath which is responsible for the regression of the residual cytoplasm, and if a contraction is involved, it would explain the coalescence of the mitochondria to a continuous spiral. It seems unlikely that the axial filament could be responsible for such changes.

Bell (1952) has suggested the utility of the freezing and drying procedure in studying small moving structures and the identification of a wave motion of the spermatid middle-piece supports the suggestion. Austin and Sapsford (1952) have described movement of the axial filament in surviving rat spermatids examined by phase-contrast microscopy. The present observations confirm this on dead stained material which was fixed intact in the testis tubule. The movement described in this paper seems to be such that the spermatid would move forwards, nucleus first.

The author's thanks are due to Prof. J. F. Danielli and Dr. J. R. Baker for criticizing the manuscript, and to the Nuffield Foundation for financial support.

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The Behaviour of the Nucleus during Spermatogenesis in *Fasciola hepatica*

By BERNARD JOHN

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With two plates (figs. 3 and 16)

SUMMARY

1. The behaviour of the nuclei of the male germ cells of *F. hepatica* L., as observed after fixation in Carnoy 6:3:1, is described and illustrated. All phases of the division and maturation of the male germ cells occur within the multi-branched and tubular testes. The daughter cells by remaining in association and dividing synchronously throughout these divisions eventually give rise to a spermatid rosette of thirty-two component cells. During spermiogenesis the nucleus of each individual spermatid cell undergoes a progressive elongation and is converted into an undifferentiated and homogeneous filiform thread.

2. The occurrence of globules of unknown origin, composition, and function is recorded within the peripheral border of spermatogonial primordia. Pycnotic primordial spermatogonia are also observed to originate in the peripheral testis cells.

3. The cyto-morphology of the chromosome complement is recorded in relation to size and the position of the attachment-constriction. The somatic number in the male cells is found to be 12 (diploid).

4. From a study of metaphase configurations chiasmata are shown to exhibit a normal distribution and there is no apparent localization of the chiasmata. The infrequent occurrence of chromatid bridges at anaphase I is correlated with the retention of an unusually large number of interstitial chiasmata.

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INTRODUCTION

SINCE the first definitive account of spermatogenesis in a digenetic Trematode (Dingler, 1910), eight major papers (Cable, 1931; Woodhead, 1931; Chen, 1937; Rees, 1939; Markell, 1943; Britt, 1947; Willey and Koulish, 1950; Willmott, 1950) have appeared in publication giving details

[Quarterly Journal of Microscopical Science, Vol. 94, part 1, pp. 41–55, Mar. 1953.]

of the process in other members of the hermaphrodite Digenea. While thes accounts generally agree, two points of interest arise in connexion wit them:

Few are complete; in particular the account of the stages of the first meioti prophase are often either incomplete (Cable, 1931; Rees, 1939; Britt, 1947 Willey and Koulish, 1950) or else absent (Woodhead, 1931; Chen, 1937 Markell, 1943). Distinct variations in detail are recorded for several of th species examined.

For these reasons and because no adequate record is available of the detaile nuclear changes in the species *F. hepatica* L. during spermatogenesis, it wa decided to study the behaviour of the nucleus in the male germ cells of th species.

TECHNIQUE

Specimens of the liver fluke, *F. hepatica*, were obtained from the livers o infected cattle (*Bos taurus*) within a period of 1 to 3 hours after the death o the cattle at the Roath Abattoir, Cardiff. On removal from the bile ducts an gall bladders the parasites were placed in a thermos flask containing mammalian Ringer's solution at a temperature of 37° C. The organisms wer transported to the laboratory in the thermos, thus ensuring a thorough washing of the external surface of the specimens. In the laboratory the animals wer carefully transferred into fresh Ringer's solution maintained at 37° C. in a incubator and from there specimens were removed for fixation within quarter of an hour of their being transferred into the fresh solution. Repeate attempts at using the special saline recommended by Stephenson (1947) fo the large-scale culture of *F. hepatica* all proved unsuccessful.

Markell (1943) has pointed out that the degree of compression employe in extending digenetic Trematodes for micro-anatomical study causes considerable internal distortion of the gonad. In the present study it was foun that if the process of fixation is carried out between two cylindrical tubes specimens are adequately fixed without suffering compression (fig. 1).

The fixative employed in the present investigation was mainly Carno 6:3:1, since it has undoubtedly given the best and the most consistent result in both trematodes (Jones, Mounts, and Wolcott, 1945; Britt, 1947; Willmott 1950) and cestodes (Jones, 1945). Other fixatives were used (Carnoy 3:1 Bouin; Sanfelice), but all gave results inferior to those obtained with Carno 6:3:1. After fixation for 1 to 2 hours specimens were washed in 90 per cent alcohol (overnight), passed through two changes of 95 per cent. alcohol (hour each), then three changes in absolute alcohol (20 minutes each), an finally cleared in two changes of benzene (20 minutes in all). Subsequen treatment in a 1:1 mixture of benzene-paraffin (1 hour) was followed by tw changes in pure paraffin (1 hour each). Finally, the specimens were embedde in pure paraffin wax of melting-point 54·5° C. This technique occasionall produced specimens which were extremely brittle; consistently better result were obtained with the ester wax technique (Steedman, 1947) and several of the later specimens were embedded in this manner with excellent results.

Sections were cut both in the horizontal plane parallel to the long axis of the body with a sledge microtome, and in the transverse plane. The horizontal sections contained many more testis tubules per section than the transverse sections and consequently permitted a complete examination of whole specimens with a far smaller number of sections. The thickness of the sections was varied for different specimens, ranging from 4 to 8 μ in the horizontal sections.

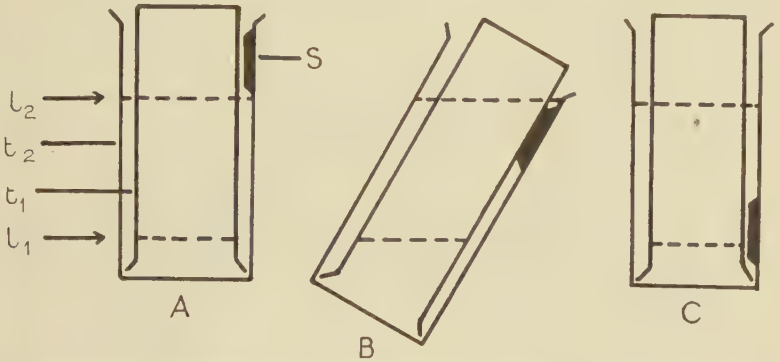

FIG. 1. Diagrammatic representation of the method of fixation. The specimen (*S*) is introduced into the space between the two tubes (t_1 and t_2) so that it lies above the level of the fixative in t_2, i.e. above l_2. When the animal is in an extended position (this can be accelerated mechanically) the tubes are tilted away from the vertical (*B*) so that the specimen is simultaneously covered with the fixative and maintained in an extended attitude by the pressure of the inner wall (t_1). In this way compression is not severe and can be relaxed by a partial or a complete return of the tubes to the vertical (*C*), when the specimen is washed into the lower volume of the fixative. Tube curvature effects can be almost completely eliminated by the use of wide diameter tubes.

Additional specimens were cut in the transverse plane at thicknesses of 6, 8, 12, and 15 μ. Staining was carried out exclusively with Heidenhain's iron haematoxylin. The haematoxylin was invariably ripened by the rapid method of Shortt (*The microtomists vade mecum*, 10th ed., p. 163); with this method of phenolic ripening an intense nuclear stain was obtained with unfailing regularity. A very faint counterstain (orange G., 5 secs.) was occasionally found useful for clarifying cytoplasmic boundaries.

All observations were made with a Zeiss one-twelfth inch oil immersion objective and a × 15 ocular. Drawings were prepared, with the aid of a camera lucida, at table level at an initial magnification of about 2,000. They are reproduced after subsequent enlargement on a horizontal projection system.

OBSERVATIONS

The structure of the testes

The gross morphology of the male reproductive system has been adequately described by Sommer (1880), but neither he nor Dehorne (1911) provides an adequate account of the histology of the testes.

The two testes each comprise a multibranched tubular system, the lobes of which contain the greater part of the germ cells arranged loosely within them, so that the interior of the gonad frequently contains open spaces. All stages of spermatogenesis are often to be observed within a single testis lobe. No significant zonation of the stages was observed except that the primordial spermatogonia occupy the peripheral layers of the testis, where they form a border of cells with a deeper staining reaction. This peripheral wall is of variable thickness, and though generally from one to several cells thick it is sometimes hardly recognizable since its component cells are widely separated. Cellular outlines in this peripheral layer are often vague owing to the crowded condition of the cells. Within the peripheral border are to be found all the remaining stages of spermatogenesis from primary spermatogonia to mature spermatozoa.

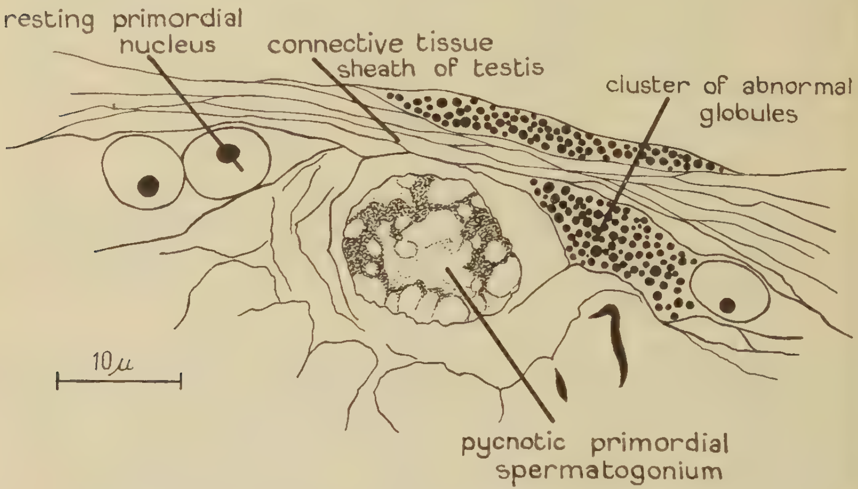
resting primordial nucleus
connective tissue sheath of testis
cluster of abnormal globules
10μ
pycnotic primordial spermatogonium

FIG. 2. Peripheral portion of a single testis lobe showing the location and structure of the two irregular components of the wall periphery.

Two components of the wall were encountered which were unlike the normal polygonal primordial cells of which the peripheral border is composed. Owing to the frequency with which both were observed, a description of them is included. The first consists of small globules which occur either singly and scattered amongst the primordial cells, or in small clusters or groups of variable size and form (fig. 2). All the specimens examined showed them, often in almost every testis lobe, and they always occurred in the wall periphery. Identical structures were described in *Dicrocoelium lanceatum* by Dingler (1910), but they do not seem to have been observed elsewhere. It is interesting to note that Dehorne (1911), while not describing the presence of the globules, appears to have figured them (pl. xi, fig. 173, Dehorne, 1911). Dingler suggested that they served as food material for the developing primordial cells,

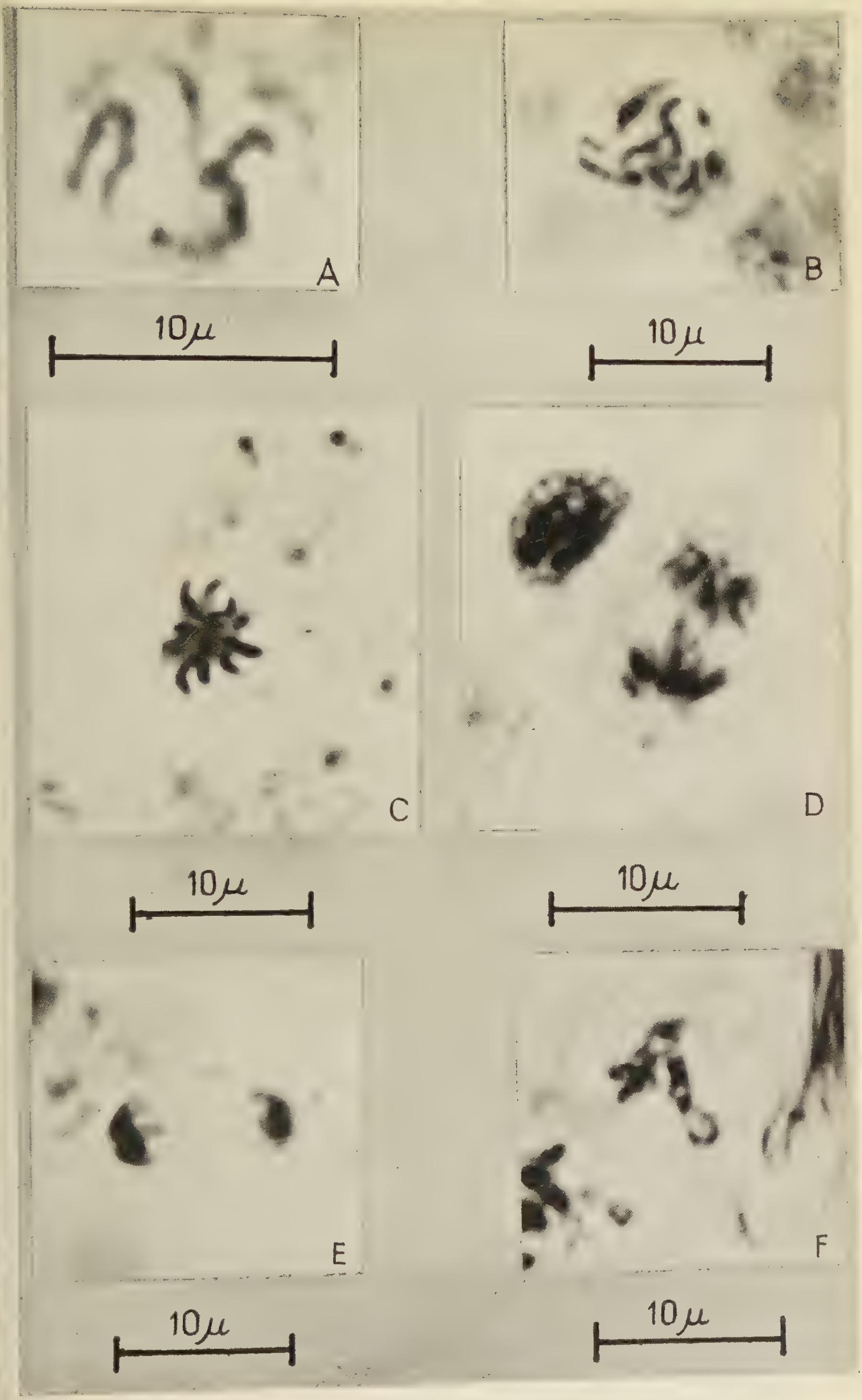

Fig. 3

B. JOHN

but in the absence of any positive evidence their formation, composition, and function remain problematical.

The second irregularity of the peripheral layer is the presence of conspicuously large structures (fig. 2) with a variable staining reaction. Bodies of like structure can also be observed occasionally lying within the testis cavity. Markell (1943) records and figures (pl. 1, fig. 24) structures both in the wall periphery and in the testis cavity which are identical with the large bodies observed in the present study. The same author also concludes that earlier workers, including Dingler, Anderson, and Rees, have almost certainly mistaken some such structures for residual cytoplasmic masses left behind after vacation of the spermatid rosettes. His conclusion is borne out in the present study, for when such structures are found in the testis cavity any relation which they may seem to exhibit with the free spermatozoa is entirely accidental. The opinion of the present author is that they are pycnotic primordial spermatogonia; their structure and mode of formation resembles closely the process of pycnosis described by Crew and Koller (1936). Chen (1937) has also observed what he regards as degenerating spermatogonia.

The spermatogonial divisions

The most common stage encountered in the cells of the peripheral testis border is that of interphase or resting stage. In *F. hepatica* the resting nucleus with its chromosomes could not be fixed satisfactorily and the chromatin network revealed by fixation is undoubtedly an artifact. No attempt is made therefore to describe or illustrate interphase nuclei either at this or at any subsequent stage during spermatogenesis.

By careful examination the nuclei of the peripheral border cells can be found to exhibit characteristic mitotic figures (fig. 3). Figs. 3, *B* and 4 show a giant pro-metaphase primordial spermatogonium encountered in the testis lobe of one individual. The cell is solitary and its enormous size can be appreciated from the resting primordial nuclei which are seen round it. No other cells of this kind were encountered elsewhere during the examination. Giant spermatogonium-like cells have been reported from the testis of young specimens of the stickleback *Pungitius tymensis* (Makino, 1935), but always in the resting condition only. Apart from this reference I have been unable to obtain any further information concerning the occurrence of giant spermatogonia.

Anderson (1935) has described the spermatogonial divisions of *Proterometra macrostoma* as anastral. Other workers (Cable, 1931; Rees, 1939; Willmott, 1950), too, have failed to observe centrioles and asters in the spermatogonial mitoses, but in no other case has the division been described as atypical. Centrioles and asters are occasionally clearly evident at this stage in some of the horizontal preparations, but in general the occurrence of central bodies is haphazard and in most cases they could not be detected in spermatogonial metaphases.

The distinction between primordial and primary spermatogonia from a cytomorphological standpoint is arbitrary. It is often difficult to identify primary spermatogonia with any degree of certainty, since they must lie very close to, and indeed probably sometimes within, the peripheral cell border from which they originate by cellular detachment. Although illustrations are not given, sufficient stages have been observed to indicate that the division of the primary spermatogonia is also a normal mitotic process resulting in the formation of two daughter cells. These remain in association instead of separating, forming a 2-celled group of secondary spermatogonia. The secondary spermatogonia in turn divide again by mitosis and the daughter cells, through remaining in association after their division, give rise to a flat plate of four cells (fig. 5), the tertiary spermatogonia. Finally, in the last of the 3 spermatogonial mitoses a 2-layered 8-celled group of associated cells is formed. Such associations constitute the primary spermatocyte groups.

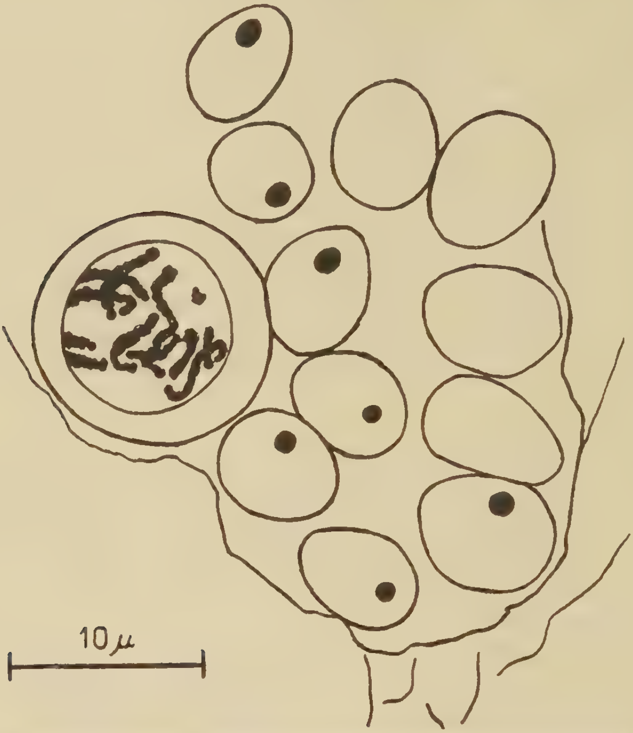

FIG. 4. A giant spermatogonial pro-metaphase from the primordial layer of a testis lobe (compare with fig. 3 B).

FIG. 3. A, mid-prophase chromosomes of the mitotic division of a primordial spermatogonium.
B, giant prometaphase primordial spermatogonium (compare with fig. 4).
C, polar metaphase in the division of a primordial spermatogonium.
D, mitotic anaphase of a primordial division.
E, primordial telophase.
F, mid-diplotene chromosomes of the first meiotic division.

The cyto-morphology of the chromosome complement

Mitotic chromosomes were studied primarily at polar metaphase of the mitoses of the primordial spermatogonia and also in metaphases of the division of the primary and secondary spermatogonia. Often the chromosomes are too crowded on the spindle for critical study and in relatively few cases could the whole chromosome complement be distinguished simultaneously. The chromosome number was found to be 12 (diploid).

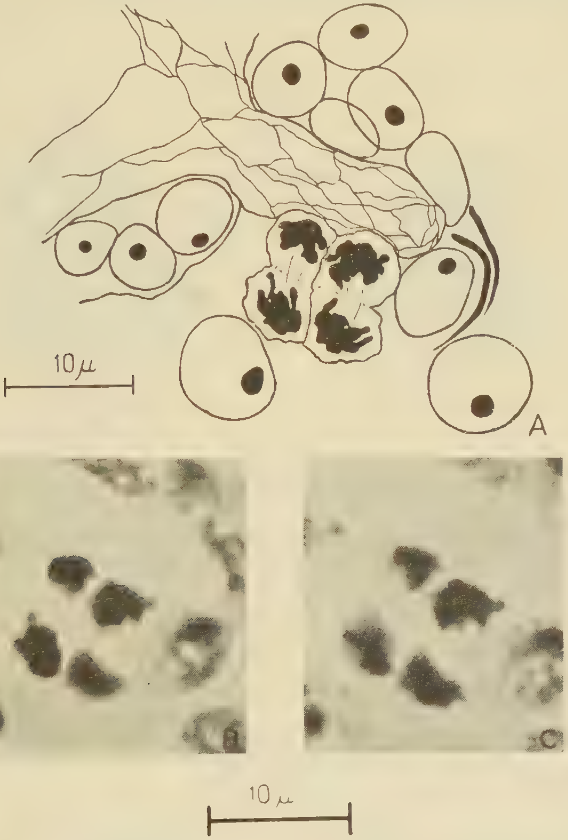

FIG. 5. Telophase of the mitotic division in a group of secondary spermatogonia.

In 1911, in a paper which seems to have evaded the attention of Anderson (1935) and Britt (1947), Dehorne records his study of the mitotic and meiotic nuclear sequences in both the male and female germ cells of *F. hepatica*. Dehorne records a diploid number of 10, two less than that given by Schubmann (1905) and Schellenberg (1911), both of whom studied oogenesis and early development of *F. hepatica*. The present study supports the observations of the two latter authors.

After an investigation of a number of mitotic plates in six distinct specimens it has proved possible, owing to the constancy of the position of the primary attachment-constriction (fig. 6) to suggest the following preliminar classification of the chromosomes for the somatic complement of the mal cells of *F. hepatica*:

1. One pair of large chromosomes with arms of equal length, and with median attachment-constriction (A.C.).
2. One pair of large chromosomes with unequal arm length and a subterminal A.C.

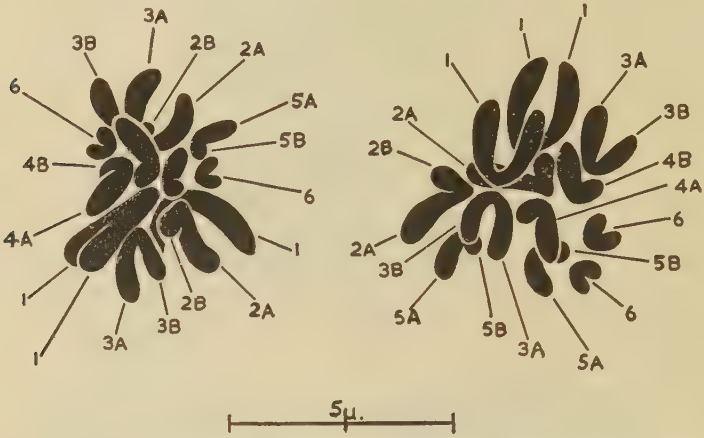

Fig. 6. Two polar metaphase plates of a primordial mitosis. Where arms of unequal length are present, these are distinguished as *A* (long arm) and *B* (short arm).

3. One pair of medium-sized chromosomes with arms of almost equal length and a submedian A.C.
4. One pair of medium-sized chromosomes with arms of unequal length and a subterminal A.C.
5. One pair of medium-sized chromosomes with arms of unequal length and a submedian A.C.
6. One pair of small chromosomes with a median A.C.

On the basis of length, therefore, the 12 mitotic chromosomes fall into three classes. There are two long pairs, three medium pairs, and one short pair. The largest pair is about three times as long as the shortest.

The spermatocyte divisions

The eight primary spermatocytes are individually the largest cells observable during spermatogenesis. Consequently a considerable growth of the daughter cells produced at cell-division must take place after the telophase o the division of the tertiary spermatogonia. The division of the nuclei of the primary spermatocytes is a normal heterotypic division. Although leptotene threads can be followed in the plane of focus, it is extremely difficult to distinguish their proximal and distal ends. It has therefore been impossible to produce a satisfactory illustration of this stage. As zygotene pairing proceeds the homologous chromosomes can occasionally be followed for considerable distances (fig. 7) and in such rare instances can be seen to correspond in chromomere structure. In no instance in the present study has it been possible to observe quadruple pachytene threads, but as chiasmata are clearly evident at diplotene (fig. 8) it seems highly probable that such a splitting must take place.

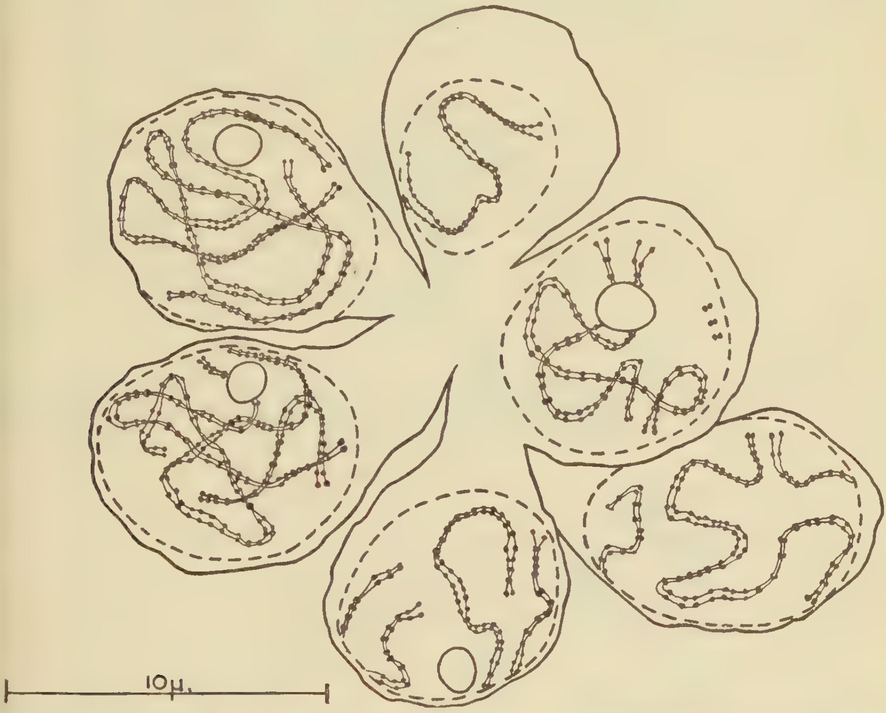

FIG. 7. Zygotene of a first meiotic prophase in a section containing six of the eight primary spermatocytes.

Moreover, at a few early diplotene configurations four threads could be counted at the point of chiasma. During the diplotene stage the tetrads continue the contraction in length begun in the pachytene stage. This contraction is accompanied by the deposition of a matrix about the individual threads resulting in the assumption of a more rounded outline (fig. 9). As a result of a relative rotation of the arms of the tetrads and a terminalization of the chiasmata, the chromosomes gradually assume a typical diakinesis appearance. At diakinesis (fig. 10) the matrix has become even more abundant, producing smoother contours about the individual chromosomes.

At the close of diakinesis six tetrads pass to the metaphase plate (figs. 16, B and C). Examination of numerous metaphase plates (e.g. fig. 11) has shown that according to the classification of Darlington (1932, p. 105) the distribution of chiasmata is normal and no localization is apparent. Generally the

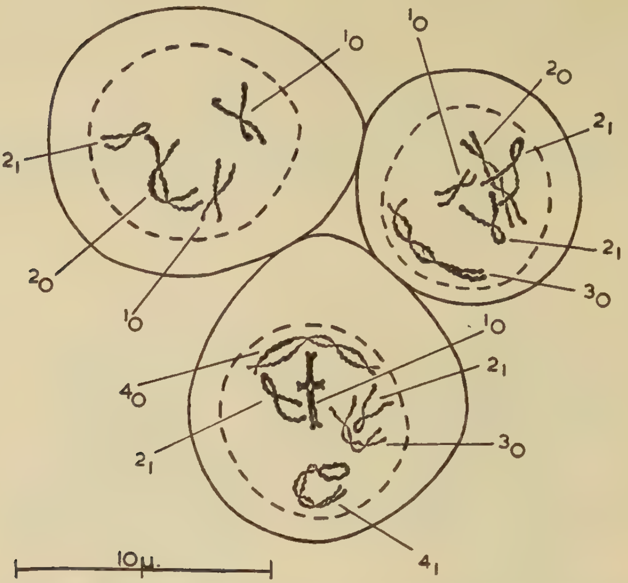

FIG. 8. Mid-diplotene of a first meiotic prophase in a section containing three of the eight primary spermatocytes. Alongside each bivalent are given the total number of chiasmata and the number that are terminal.

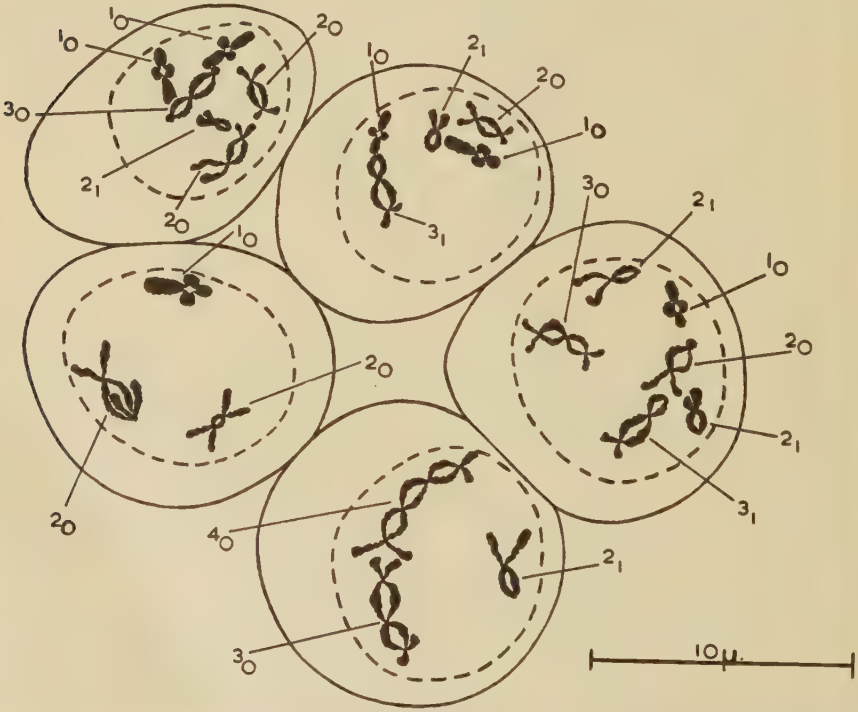

FIG. 9. Late diplotene in a group of five primary spermatocytes. Alongside each bivalent are given the total number of chiasmata and the number that are terminal.

bivalents segregate more or less simultaneously at the first meiotic anaphase, but in four anaphasic spermatocytes, occurring in three distinct individuals, two dyads have been observed to remain connected by a chromatid bridge (fig. 16, D). The production of anaphasic bridges may be ascribed either to

1. the presence of an inversion; since crossing over in an inverted segment would give rise to a chromatid bridge and fragments; or
2. the presence of a high number of interstitial chiasmata being retained in one particular tetrad at metaphase; so resulting in a delaying of the separation of the associated dyads.

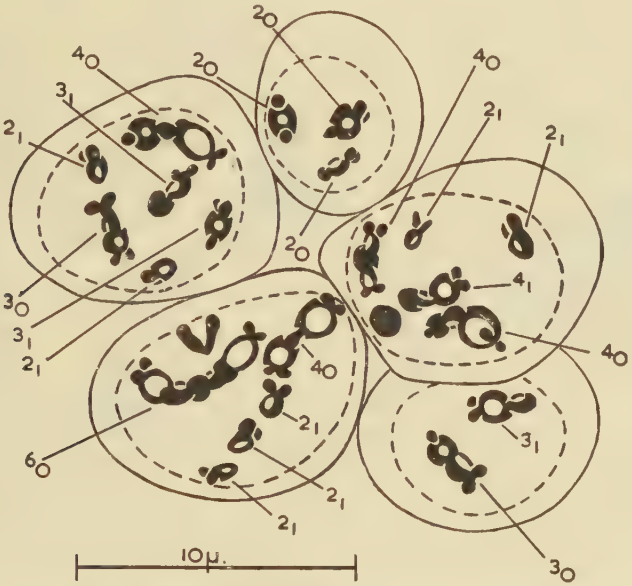

FIG. 10. A group of five primary spermatocytes with the nuclei at diakinesis. Alongside each bivalent are given the total number of chiasmata and the number that are terminal.

The complete absence of fragments near the bridge in all four cases, and the extremely rare incidence of such bridges, suggests that the lagging configuration is due to the second of the two alternatives. Similar behaviour has been reported by Koller (1936) and Ahmed (1940).

After telophase of the first meiotic division the chromosomes reconstitute into daughter nuclei which must pass into a fairly complete interphase condition, for at the ensuing prophase of the second division the chromosome threads are considerably longer than the contracted meta- and anaphase chromosomes of the first division. With the completion of the first meiotic division and the division of the eight cytoplasmic portions, a 16-celled cluster is formed, each individual cell of which is a secondary spermatocyte. Although division is again complete, the component spermatocytes of a group remain in contact with one another.

Division of the nuclei of the secondary spermatocytes completes the meioti distribution of the chromosomes. Examination of prophase II chromosome shows that the individual threads are visibly double at certain places from thei first appearance. This duality of the chromosome dyads is also clearly eviden at metaphase II (fig. 12). The end of the second telophase results in th production of 32 nuclei, which together with their associated and componen cytoplasmic masses constitute a spermatid rosette.

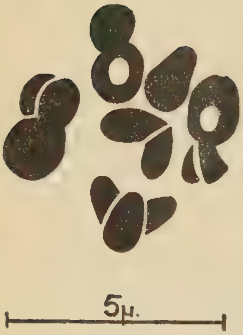

FIG. 11

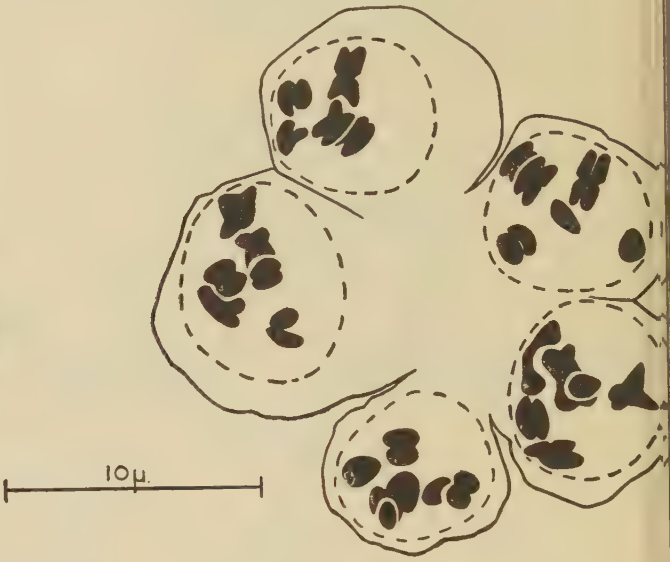

FIG. 12

FIG. 11. Polar metaphase in the first meiotic division of a primary spermatocyte.
FIG. 12. Second meiotic metaphase in a group of five of the sixteen secondary spermatocytes.

Nuclear changes during spermiogenesis

The spherical interphase nuclei of the spermatid cells become ovate and exhibit a characteristically weak staining reaction. The ends of the nuclei towards the periphery of the cytoplasmic wall appear to begin protruding through the cell wall. Whether the wall is ruptured or forms a thin enclosing membrane is not clearly discernible. The main growth of the nuclei at this phase, however, appears to be directed towards the centre of the rosette, for it is mainly by an elongation in this direction that the spermatid nuclei first increase in length. During the initial part of this process of nuclear elongation the staining reaction is still characteristically weak. At a slightly later stage (fig. 13) the nuclei take up the stain rapidly and appear as homogenous and apparently undifferentiated bodies. With the assumption of coiling by the elongated nuclei (fig. 14) the peripheral portions of the nuclei begin to re-orientate so that they all come to lie pointing in one direction (fig. 15). At the completion of the coiling process the nuclei start to uncoil again and so force

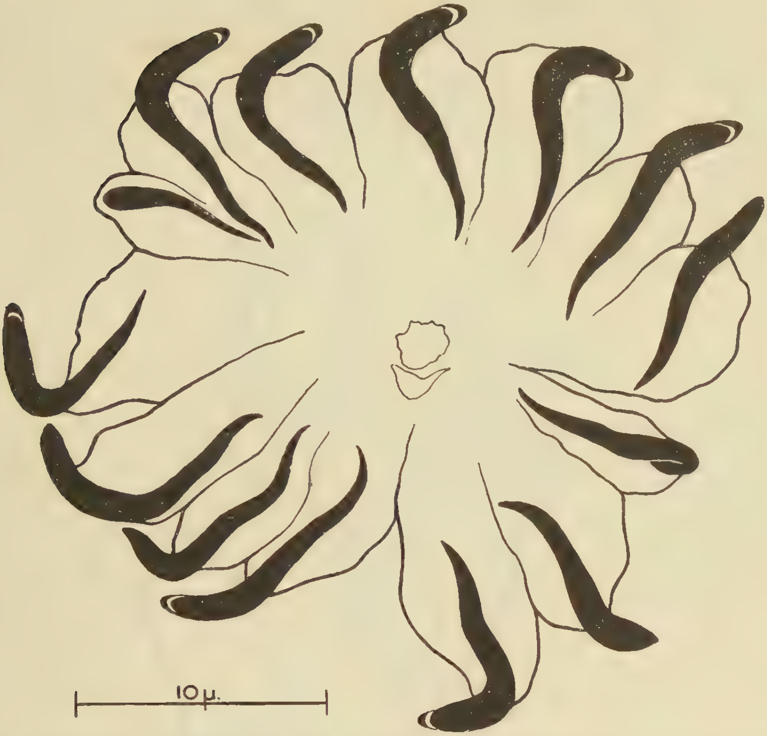

FIG. 13. Fourteen of the thirty-two cells of a spermatid rosette with the nuclei in centrifugal elongation.

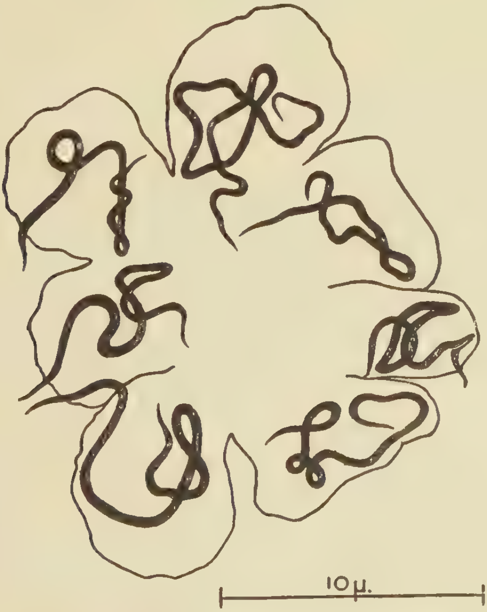

FIG. 14. A section of a spermatid rosette, showing seven of the cells with the elongate nuclei exhibiting coiling.

the peripheral portions of the nuclei centrifugally out of their cell bodies. A the nuclei are all orientated in the same direction peripherally they emerge a a bundle of filiform threads, leaving behind an evacuate cytoplasmic rosette Eventually the individual threads of an emergent cluster separate and come t

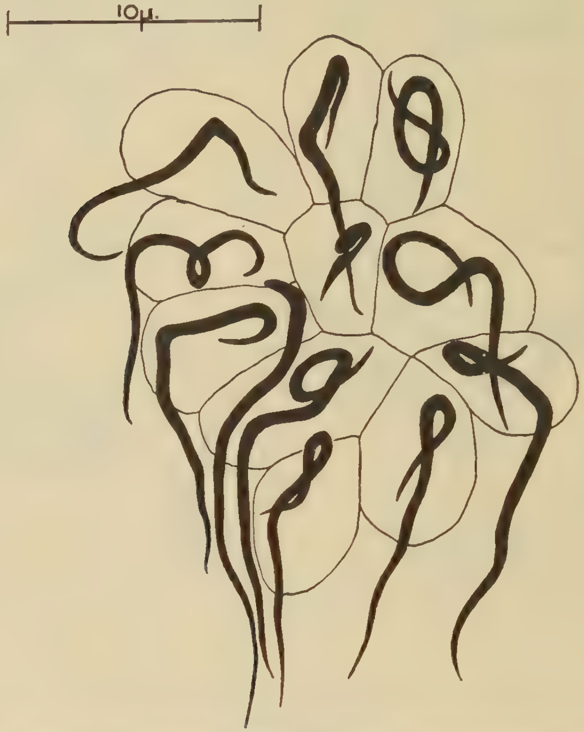

FIG. 15. Surface view of a spermatid rosette to show the peripheral reorientation of th spermatid nuclei.

lie free in the loose cavities of the gonad; from here they pass to the va deferens and subsequently to the seminal vesicles.

Whether the filiform structures produced by the elongation of the spermati nuclei are to be regarded as entire spermatozoa cannot be confidently answere from the present study. Most earlier workers have assumed that the spermatozoon of digenetic trematodes is entirely nuclear in origin, basing thei assumption on observations made usually after the use of nuclear fixatives

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FIG. 16. A, late diplotene configurations.

B, polar view of a first meiotic metaphase plate showing the six tetrads.

C, polar view of a group of three primary spermatocytes with the nuclei at the metaphas stage of the first meiotic division.

D, first meiotic anaphase showing chromatid bridges formed by lagging.

E, part of a spermatid rosette with the elongate spermatid nuclei exhibiting coiling. A singl late diplotene bivalent can be seen to the left of the rosette.

Such fixatives might well destroy delicate cytoplasmic structure or impede observation of it. It seems hardly justifiable to conclude on this basis that the spermatozoon is without any cytoplasmic component whatsoever and in the present account the matter is left open. The problem becomes more acute in view of the fact that Severinghaus (1928) claims to have observed cytoplasmic flagella in Benda preparations of the developing spermatozoa of the dioecious trematode *Schistosoma japonicum*, although iron haematoxylin preparations of material fixed in the same way failed to show them. Woodhead (1931) also claims to have observed a cytoplasmic tail in the spermatozoa of the trematode family Bucephalidae.

This investigation forms a section of a thesis approved for the degree of M.Sc. of the University of Wales, and was carried out during the tenure of a Dr. Samuel Williams Research Scholarship of the University of Wales.

I wish to express my thanks to Prof. J. Brough under whose supervision the work has been carried out. I am indebted to Mr. J. H. M. Hughes, Veterinary Officer at the Roath Abattoir, Cardiff, for his kindness in allowing me to collect specimens of flukes from cattle slaughtered there. Finally, I would like to thank Mr. D. A. Erasmus for his advice in the production of the photomicrographs.

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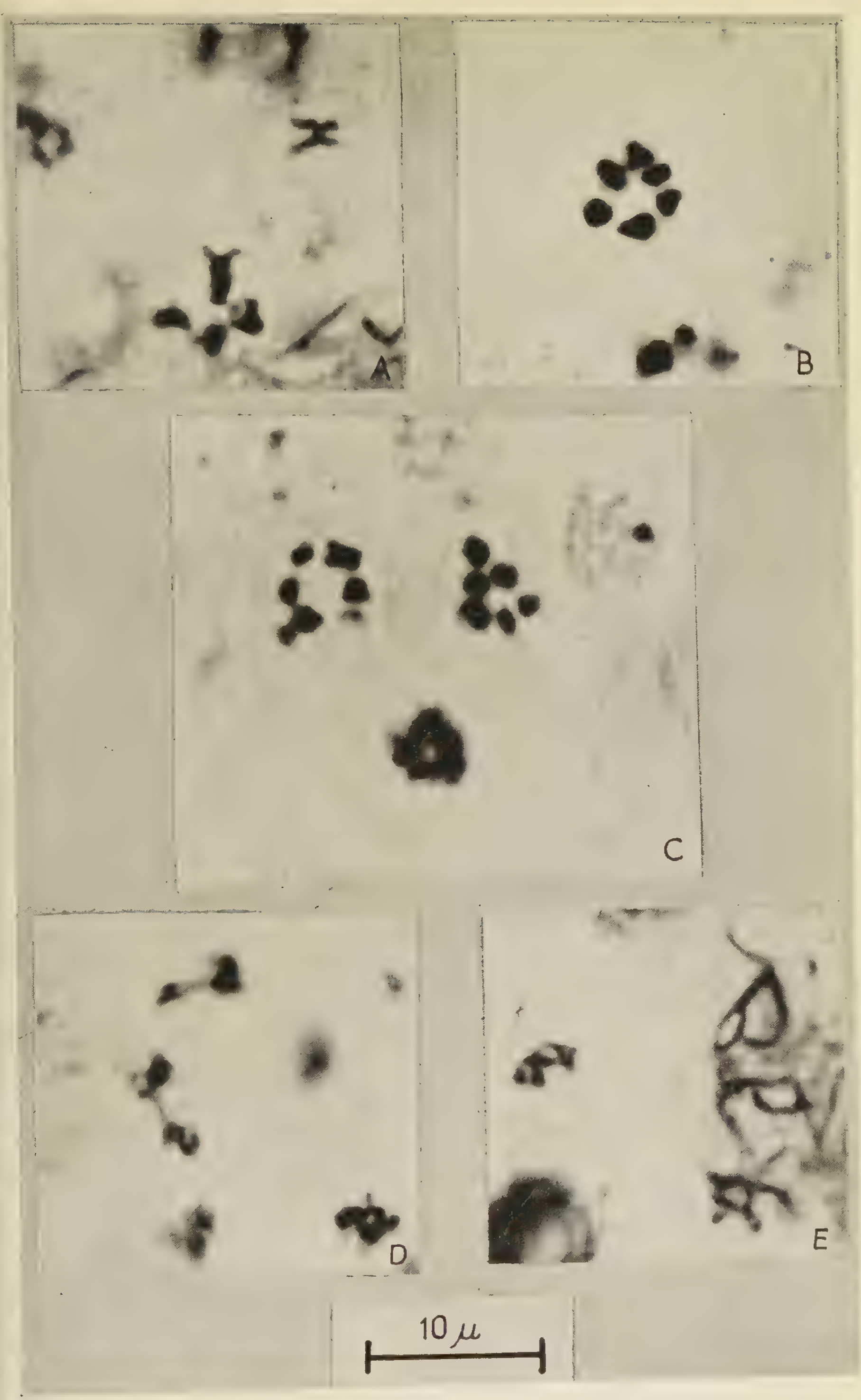


Fig. 16

B. JOHN

The Shell in the Lamellibranchia

By G. OWEN

(*From the Department of Zoology, University of Glasgow*)

SUMMARY

1. In the Lamellibranchia, the direction of growth at any region of the valve margins may be resolved into: (*a*) a radial component radiating from the umbo and acting in the plane of the generating curve; (*b*) a transverse component acting at right angles to the plane of the generating curve; (*c*) a tangential component acting tangentially to, and in the plane of, the generating curve. The radial component is always present and affects the form of both valves while the transverse component may be reduced or absent in one valve.

2. The lamellibranch mantle/shell is orientated with reference to the normal axis. This normal axis follows that sector of the shell secreted by the normal zone of the mantle edge (i.e. where the effect of the transverse component is greatest) and passes through the umbo, the normal zone and the point at which the greatest transverse diameter of the shell intersects the surface of the valves.

3. The form of the shell valves should be considered with reference to: (*a*) the outline of the generating curve; (*b*) the spiral angle of the normal axis; (*c*) the form (i.e. plano-spiral or turbinate spiral) of the normal axis.

4. The 'deflection' anteriorly of the umbones and the splitting of the anterior portion of the ligament in many bivalves is a consequence of a tangential component affecting the form of the valves.

5. In both gastropods and lamellibranchs, the turbinate spiral shell is the resultant of two differential growth ratios. In the Gastropoda, however, both ratios act perpendicularly to the plane of the generating curve, while in the Lamellibranchia, one acts perpendicularly to this plane while the other acts *in* the plane of the generating curve.

INTRODUCTION

THIS present work has arisen from a study of shell-form in *Glossus humanus* (*Isocardia cor*) and a comparison of this species with other members of the Lamellibranchia. The remarkable variation in shell-form in the Mollusca is well known but whereas the Gastropoda have received considerable attention from many workers the Lamellibranchia have been largely neglected. Lison (1949) has recently reviewed the scanty literature.

The majority of investigators have considered the lamellibranch shell in terms of 'length', 'breadth', and 'height'. These characters alone do not provide a satisfactory means of comparing shell-form in different species. It is hoped, therefore, that this present work will provide a convenient method of analysing and comparing shell-form in different species of Lamellibranchia.

[Quarterly Journal of Microscopical Science, Vol. 94, part 1, pp. 57–70, Mar. 1953.]

SHELL-FORM

Typically, the lamellibranch shell consists of two valves linked dorsally b a ligament, and growth results from marginal increment. Viewed anteriorly each of the valves grows as a logarithmic spiral surface about a fixed poin which coincides with the umbo (D'Arcy Thompson, 1942). The genera

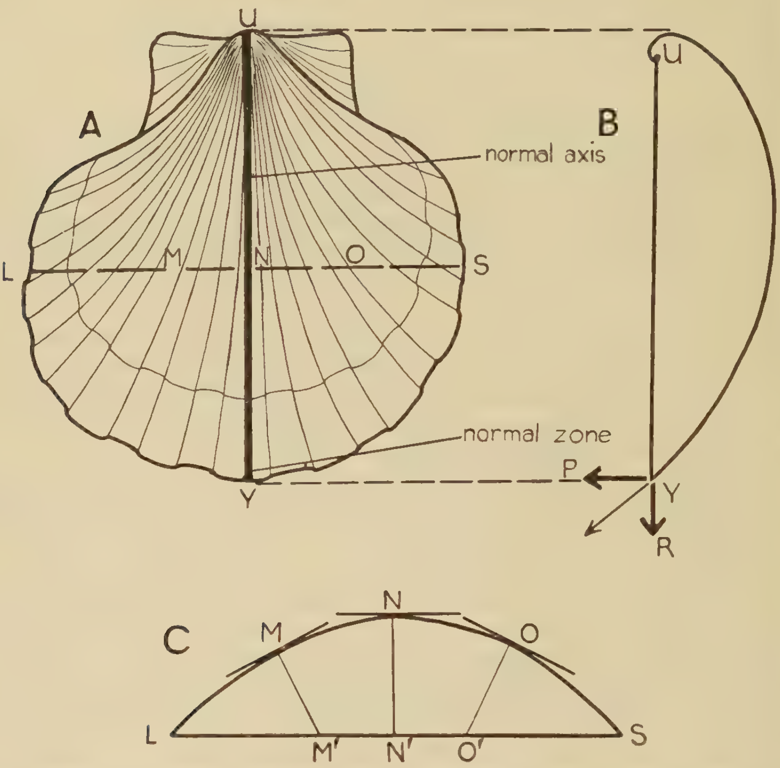


FIG. 1. Diagrams to explain the terms used in the text. A, right, concave valve of *Pecte* *maximus*; B, a section through *UY*; C, a section through *LNS*. *P*, transverse component *R*, radial component; *UY*, normal axis; *Y*, normal zone. (See also text.)

mathematical properties of the logarithmic spiral and their application to shell growth in lamellibranchs are discussed by Lison (1949). Marginal increase o the valves results from the activity of the mantle edge which is thus responsible for the form of the shell. The form of the shell in the primitive bivalve was probably not unlike that of *Glycymeris* but for convenience the ribbed shell of *Pecten maximus* is taken as a starting-point in this discussion. (The symmetry of the shell in *Pecten* is not comparable with that of *Glycymeris*. In *Pecten*, there is a secondary symmetry due to loss of the anterior adducto muscle and subsequent reorganization round the posterior adductor (Yonge, 1953).) Each of the ribs represents a sector of the shell which, during growth, has been secreted by the same, ever widening, region of the mantle edge. The direction of growth at any region of the mantle edge may be resolved into a transverse component (fig. 1, B, P) acting at right angles to the plane of the generating curve (represented by the valve margin) and a radial component (R) radiating from the umbones and acting in the plane of the generating curve (Owen, 1952). The ratio of these two components determines the spiral angle and so the concavity of the valves; e.g. growth of the flat valve in *Pecten maximus* is the result of the radial component alone.

In *Pecten*, the central rib of the concave valve coincides with that sector of the valve which exhibits the greatest concavity (Gewolbescheitelbahn of Haas, 1933). This is demonstrated in fig. 1, C which represents a section through the valve at LS (fig. 1, A). $N'N$, $O'O$, and $M'M$ are drawn perpendicular to the tangents to the surface of the valves at N on the central rib and O and M on ribs anterior and posterior to the central rib respectively. $N'N$ is perpendicular to the plane of the generating curve, represented by the line LS, indicating that N is the turning-point of the curve LNS. Since the central rib coincides with the sector of the shell exhibiting the greatest concavity, it follows that the effect of the transverse component is greatest at that region of the mantle edge which secretes the central rib. Provided the generating curve is in one plane as in *Pecten* (i.e. unlike the gaping Myidae and Pholadidae) it is proposed to designate this region (fig. 1, A, Y) as the 'normal zone' of the mantle edge and a line (UNY) drawn along the centre of the sector of the valve exhibiting the greatest concavity as the 'normal axis' of the lamellibranch mantle/shell (Yonge, 1952). Thus, the normal axis can be considered with reference to three points: the umbo, the normal zone of the mantle margin, and the point at which the greatest transverse diameter of the shell intersects the surface of the valves.

In *Pecten*, the normal axis lies in one plane, i.e. it is a plano-spiral (fig. 1, B, UY). Enlargement of the generating curve with growth results in the ribs anterior and posterior to the normal axis being increasingly displaced away from the normal plane; unlike the normal axis they are turbinate spirals. Similarly, in *Glycymeris* (fig. 2, A), the normal axis is a plano-spiral and each valve would, if a complete whorl were produced, describe a plano-spiral about the umbo. But these are both genera in which growth of the mantle/shell is the resultant exclusively of radial and transverse components. In many bivalves, e.g. Mytilidae and Veneridae, the direction of growth at any region of the mantle margin may be resolved into three components. In addition to the radial and transverse components already discussed, there is a third component acting tangentially to, and in the plane of, the generating curve (fig. 2, D, T). Due to this tangential component the normal axis is no longer a plano-spiral but a turbinate spiral and in this case, each of the valves would, if a complete whorl were produced, describe a turbinate spiral about the umbo (e.g. as in *Glossus humanus*, fig. 2, D). While the radial and transverse components tend to produce a plano-spiral about the umbo in a plane perpendicular to the plane of the generating curve, the radial and tangential components tend to produce a plano-spiral in the plane of the generating curve. The form of the valves may be considered as the resultant of these two systems of spirals.

Perhaps the most interesting example of the effect of this tangential component is to be found in a comparison of two members of the Ostracea, namely, *Gryphaea* and *Exogyra* (fig. 3). In *Gryphaea*, growth of the flat right valve is due almost entirely to the radial component and results in a flat or even convex valve. Growth of the concave left valve is the resultant of radial and transverse components. The spiral angle is high, the normal axis in one plane, and the valve markedly globular with a swollen, overhanging, umbonal region. Growth of the shell of *Exogyra*, on the other hand, is considerably modified by a tangential component (fig. 3, B). Marginal increase of the flat right valve is the resultant of radial and tangential components; the transverse component being much reduced or absent. The valve has the form of a flat spirally coiled plate. The form of the concave left valve is the resultant of all three compo-

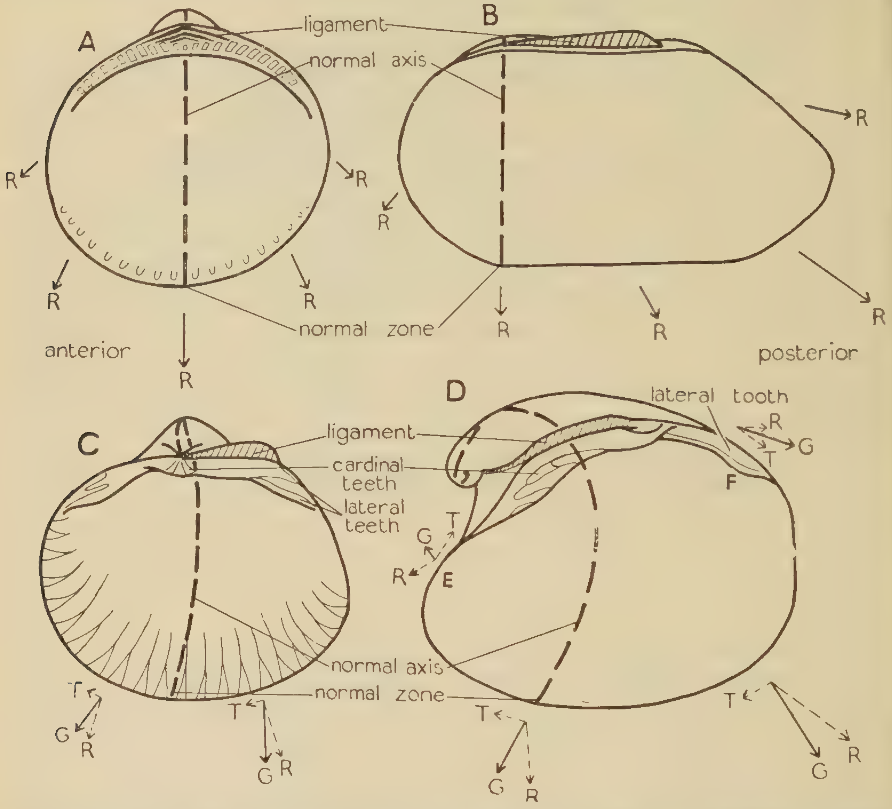


FIG. 2. Diagrams of the left valves of: A, *Glycymeris*; B, *Anodonta*; C, *Cardium*; D, *Glossus*. *G*, the direction of growth at the mantle/shell edge in *Cardium* and *Glossus*; *R*, radial component; *T*, tangential component. The transverse component acting at right angles to the plane of the page is not shown.

nents, radial, transverse, and tangential, so that the normal axis is a turbinate spiral. In comparison with *Gryphaea* it is interesting to note that the umbonal region of the concave valve of *Exogyra* does not overhang the upper valve. In valves possessing a high spiral angle and no tangential component, there is a tendency towards 'locking' of the valves due to the apposition of the swollen umbonal regions; e.g. in *Gryphaea* and many of the Cardiacea where the tangential component is so small as to have little effect on the form of the mantle/shell. In those bivalves in which the tangential component is great enough, the umbones are directed anteriorly as in the equivalve species *Glossus humanus* (fig. 2, D). In this species, the swollen umbonal regions lie below the hinge axis (Yonge, 1953) and do not prevent the opening of the valves.

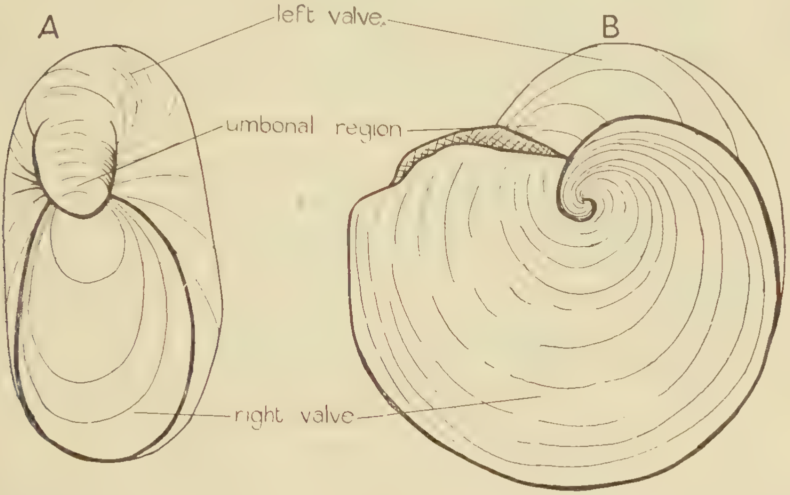


FIG. 3. A, *Gryphaea*; B, *Exogyra*.

In *Glycymeris*, the normal zone is mid-ventral and coincides with the region of greatest marginal increment (fig. 2, A). Moreover, both marginal increment and the effect of the transverse component decrease symmetrically on each side. Thus, the valves are symmetrical about the normal axis. The normal zone, however, need not coincide with the region of greatest marginal increase. For instance, in *Gari tellinella* or *Siliqua patula* (Yonge, 1952), the generating curve is an ellipse which is roughly symmetrical about the normal axis. There are two regions of maximum marginal increase symmetrically disposed at each end of the ventral margin. The effect of the transverse component, however, remains greatest mid-ventrally; it is the radial component which produces the greater growth at the two ends of the shell.

In many genera the outline of the generating curve is asymmetrical about the normal axis. This is the case irrespective of whether the region of greatest marginal increase is anterior or posterior to the normal zone. Marginal increase is generally greatest posteriorly and it is the posterior region of the shell which is enlarged. Thus in *Anodonta* (fig. 2, B) and *Ensis* (Yonge, 1952) maximum marginal increase takes place posteriorly but the greatest effect of the transverse component remains ventral to the umbo as in *Glycymeris* and *Gari*. It follows that the normal axis is a plano-spiral and each valve would, if a complete whorl were produced, describe a plano-spiral about the umbo.

The normal axis of the mantle/shell offers a convenient means of comparing shell-form in different species of Lamellibranchia. The spiral angle of the normal axis is a measure of the concavity of the valves while the form (i.e. plano- or turbinate spiral) of the normal axis is correlated with the form of the valves and the orientation of the umbones. The tangential component acts clockwise when viewed from the left side as in fig. 2, D. Due to this tangential component the umbones come to be directed anteriorly (in *Nucula*, the tangential component acts anti-clockwise when viewed from the left side; the umbones are directed posteriorly).

Yonge (1952) has described the use of radial co-ordinates as a means of comparing shell-form in lamellibranchs. In those genera in which the form of the valves is the resultant of radial and transverse components and thus the normal axis a plano-spiral, e.g. *Glycymeris* and *Ensis*, the valves may be represented by straight line radial co-ordinates. But in those genera in which the form of the valves is affected by a tangential component and thus the normal axis a turbinate spiral, e.g. as in *Mytilus*, *Venus*, and *Glossus*, the straight line radial co-ordinates are transformed into a system of spiral co-ordinates.

The Effect of Shell-form on the Hinge

The mantle in the Lamellibranchia is laterally compressed and bilobed, the two lobes remaining connected dorsally by a narrow neck of mantle tissue. In preference to the term 'pallial suture' which has been frequently used to describe this region it is proposed to use the term 'mantle isthmus'.

In genera where the tangential component is absent (e.g. *Tellina*), the cardinal teeth radiate across the hinge plate increasing slowly in all dimensions with growth. Anterior and posterior to the mantle isthmus, where the radial component increases and acts obliquely to the mantle/shell edge, the lateral teeth are variously elongated. In genera such as *Arctica* (*Cyprina*) and *Glossus*, however, the mantle isthmus is continuously displaced posteriorly owing to the effect of the tangential component on the mantle edge (fig. 2, D, *T*). Thus, in *Glossus humanus* (fig. 2, D), the cardinal teeth extend posteriorly and are long and almost parallel to the sides of the hinge plate. The anterior lateral tooth is not present while the posterior lateral is elongated since in this region the tangential component, by displacing the secreting surface posteriorly, reinforces the effect of the radial component.

The tangential component also has an indirect effect on the anterior region of the ligament. When there is no tangential component affecting the form of the valves, as in *Glycymeris* (fig. 2, A) and *Anodonta* (fig. 2, B), growth of the mantle/shell anterior to the umbo takes place anteriorly, i.e. away from the umbo. In *Glossus humanus*, on the other hand, growth of the valves is affected y a tangential component. As shown in figure 2, D, the radial (R) and tangential omponents (T) anterior to the umbones are opposed. Owing to the greater nagnitude of the tangential component at this region, growth of the mantle/ hell margin takes place in a posterior direction between the umbones causing them to be increasingly separated as growth proceeds. This increasing separation of the umbones results in the progressive splitting of the anterior portion of the ligament. The mantle isthmus is displaced posteriorly and thus the ligament extends more rapidly in this direction than would be the case if there were no tangential component. The effect of this type of growth on the ligament in *Glossus humanus* is shown diagrammatically in fig. 4. The ligament, bounded by the lines OX and OZ, extends posteriorly during growth. The

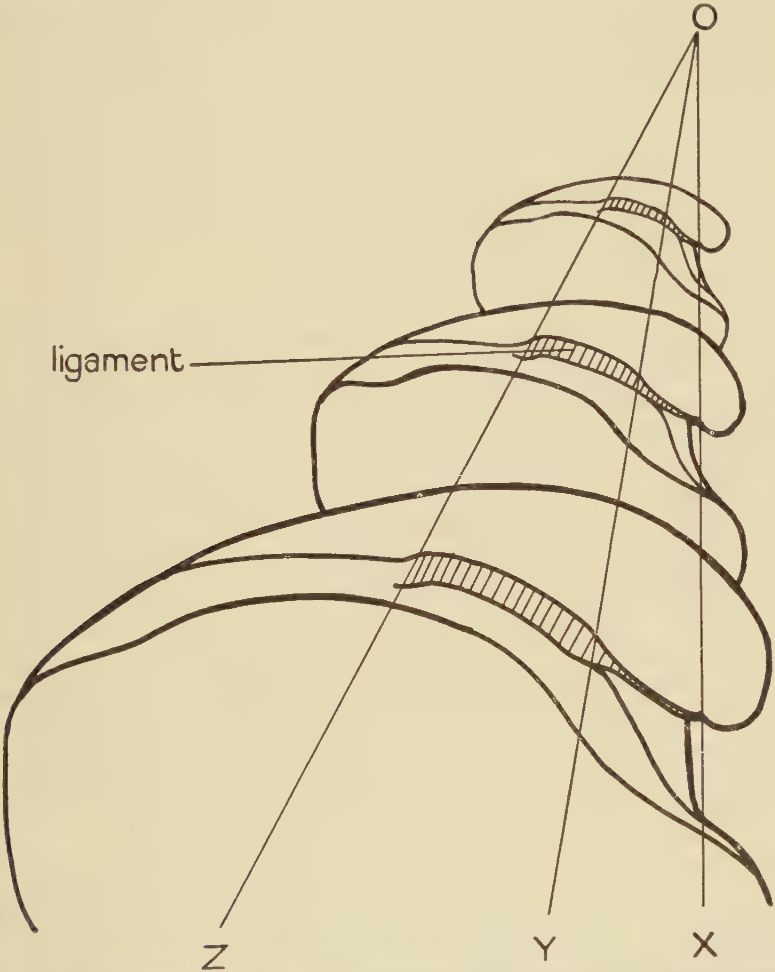


FIG. 4. Diagram representing three stages in the growth of *Glossus humanus* and illustrating the progressive splitting of the anterior portion of the ligament.

tangential component causes the mantle shell edge 'anterior' to the umbone to extend posteriorly between them and results in the anterior portion of th ligament, bounded by the lines OX and OY, being increasingly split. Thu the intact ligament, bounded by the lines OZ and OY, shifts posteriorl during growth.

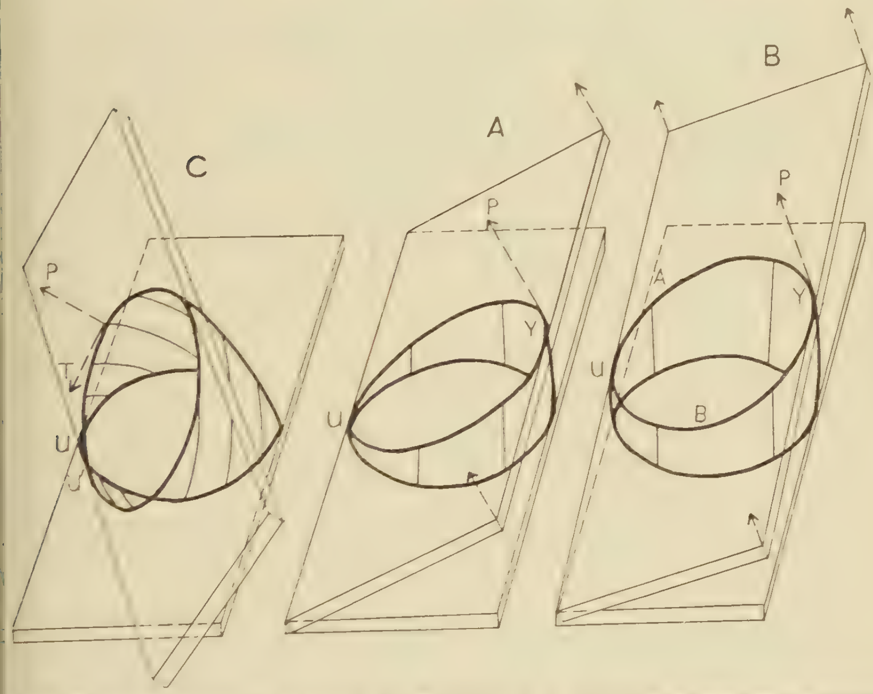
It would appear that growth of a turbinate-spiral shell due to a tangenti component results in a reduction in efficiency of the ligament. Naturally, th split anterior portions of the ligament will cease to function, while Dall (189 has pointed out that no bivalve ligament can function around a curve. Yet i those genera such as *Glossus*, *Exogyra*, and *Diceras* in which shell-form i affected by a tangential component, the ligament is curved; the degree c curvature appears to be correlated with the magnitude of the tangential com ponent. Certainly, in adult forms of *Glossus humanus*, the intact ligamen appears to produce little or no opening thrust.

The growth of many bivalves is affected by a tangential component bu rarely to such an extent or coupled with such a high spiral angle as in *Glossu humanus*. In *Cardium edule*, the tangential component is so small as to hav little effect on the form of the valves, but, nevertheless, the normal axis is turbinate spiral (fig. 2, C). In *Modiolus*, the tangential component is more pro nounced and, as in *Glossus humanus*, has resulted in splitting of the anterio portion of the ligament. The fossil genus *Hippopodium*, belonging to th Modiolopsidae, shows an even greater degree of splitting of the ligament Trueman (1950) considered the splitting of the anterior region of the ligamen in *Mytilus edulis* to be due to the increasing gape, with growth, of the 'earlie valves'. He observed, however, that the split portions of the ligament becam embedded in each valve. This 'embedding' of the anterior portions of th ligament is due to the posteriorly directed growth of the mantle shell immediately 'anterior' to the umbones. There can be little doubt that the splitting of the ligament in *Mytilus edulis* is comparable with that in *Glossu humanus* and is due to a tangential component affecting the form of the mantle shell.

The Turbinate-spiral Shell in Gastropods and Lamellibranchs

The plano-spiral shell in gastropods, e.g. *Planorbis*, is due to growth of the mantle shell edge out of the plane of the generating curve, as it exists at any moment, being greatest at one margin, least at the opposite margin and intermediately graded around both sides of the mantle shell edge (Huxley, 1932). This is represented diagrammatically in fig. 5, A (the radial component is not shown). Growth out of the plane of the generating curve is least at U and greatest at Y. Huxley (1932, p. 155) describes this differential growth at U and Y as a median differential growth ratio. If at A, as shown in fig. 5, B, there is a secondary increase in growth out of the plane of the generating curve and at B, a secondary decrease, the resultant will be a turbinate-spiral shell as exemplified by *Helix* and *Turritella*. Huxley describes this as a lateral differential growth ratio. Such a shell may be considered as the resultant of two ystems of plano-spirals inclined at an angle to one another and both per-endicular to the plane of the generating curve.

In those bivalves in which the normal axis is in one plane, each valve would, a complete whorl were produced, describe a plano-spiral about the umbo. he median differential growth axis lies in the plane of the normal axis; the ffect of the transverse component being greatest at the normal zone of the



IG. 5. Diagrams attempting to explain the formation of: A, a plano-spiral shell; B, a turbinate-piral shell in the Gastropoda; C, a turbinate-spiral valve in the Lamellibranchia. The radial component is not shown.

nantle shell (fig. 5, A, *Y*). Thus, in genera with equilateral valves such as *Pecten* nd *Glycymeris* and also in those with inequilateral valves such as *Anodonta* nd *Ensis*, plano-spiral valves are produced. These are similar to the plano-piral shell in gastropods in that they are the result of a median differential rowth ratio.

Huxley (1932), D'Arcy Thompson (1942), and Lison (1949) considered hat the turbinate-spiral shell in lamellibranchs, as exemplified by *Glossus umanus*, was similar to the turbinate-spiral shell in gastropods. In the Lamelli-ranchia, however, growth of a turbinate spiral-shell is complicated by the xistence of two valves and a connecting ligament. The shell in gastropods nay be considered as the resultant of variations of the transverse and radial omponents around the mantle shell margin so as to produce two differential rowth ratios. The turbinate-spiral shell in lamellibranchs is due to the existence of a third component acting tangentially to the mantle/shell edge Anterior to the umbones, the radial and tangential components oppose on another and there is a region at the mantle/shell edge where they are more o less equal (fig. 2, D, E). Marginal increment at this region is due almost entirel to the transverse component. Posterior to the umbones, the radial and tangential components augment one another (F). A lateral differential growth rati is thus produced which, coupled with the median differential ratio, results i the production of a turbinate-spiral shell (fig. 5, C). As in gastropods, growth o a turbinate-spiral shell is the resultant of two differential growth ratios, bu whereas one (the median ratio) acts perpendicular to the plane of the generatin curve, the other (the lateral ratio) acts in the *same* plane as the generating curve

Huxley's hypothesis of growth gradients has been criticized by Lison (1949) Fig. 6, A represents Lison's interpretation of Huxley's hypothesis explainin the formation of a plano-spiral shell. The diagram represents the distributio of forces at all points on the generating curve. 'L'équipotentielle correspondant à l'activité zéro est l'axe $U'U''$' and this secretory activity increase linearly along the parallel lines $U'X$, UY, and $U''Z$. Similarly, fig. 6, B represents the formation of a turbinate-spiral shell. U^1U^2 represents 'the axis o zero activity' while a median differential growth ratio is represented along th line U^1Y and a lateral differential growth ratio along the line U^2L. Liso points out that the distribution of forces represented in fig. 6, A will resul in the material secreted at Y having the form of a circle and not a logarithmi plano-spiral. The distribution of forces represented in fig. 6, B will als result in the formation of a circular structure but with the axis of revolutio now forming an angle with the line U^1Y; the magnitude of the angle will b dependent on the value of the lateral differential ratio.

The error, however, would seem to lie not in Huxley's hypothesis but i Lison's interpretation of this hypothesis. In both diagrams the radial component has been omitted. The value of this is greatest at Y and decreases aroun both sides of the generating curve to a minimum at C. This radial componen is shown in fig. 7, A (R) and it will be seen that the resultant of radial and transverse components or, according to Huxley, 'length growth and width growth' will be a logarithmic plano-spiral and not a circle. Similarly, in fig. 6, B, a radial component will result in the formation of a logarithmic spiral. Thi spiral, however, will be a plano-spiral and not a turbinate-spiral and reveal another misunderstanding in Lison's presentation.

Lison considered the growth forces of the lamellibranch mantle/shell to be parallel to one another.

> La 'matrice' sécrétant le calcaire des coquilles est le siège d'un champ d'activit disposé comme suit: les lignes d'égal potentiel d'activité sont toutes parallèles entr elles et par conséquent les lignes de force de ce champ, toutes perpendiculaires au équipotentielles, sont aussi toutes parallèles entre elles.

This is certainly not so. The lines of growth of the lamellibranch mantle/shel *radiate* from a node of zero or minimum growth represented by the umbo. I s for this reason that radial co-ordinates may be used in comparing the valves of lamellibranchs (Yonge, 1952). If the diagram shown in fig. 6, B is reconstructed as in fig. 7, B, it will be seen that the existence of two differential

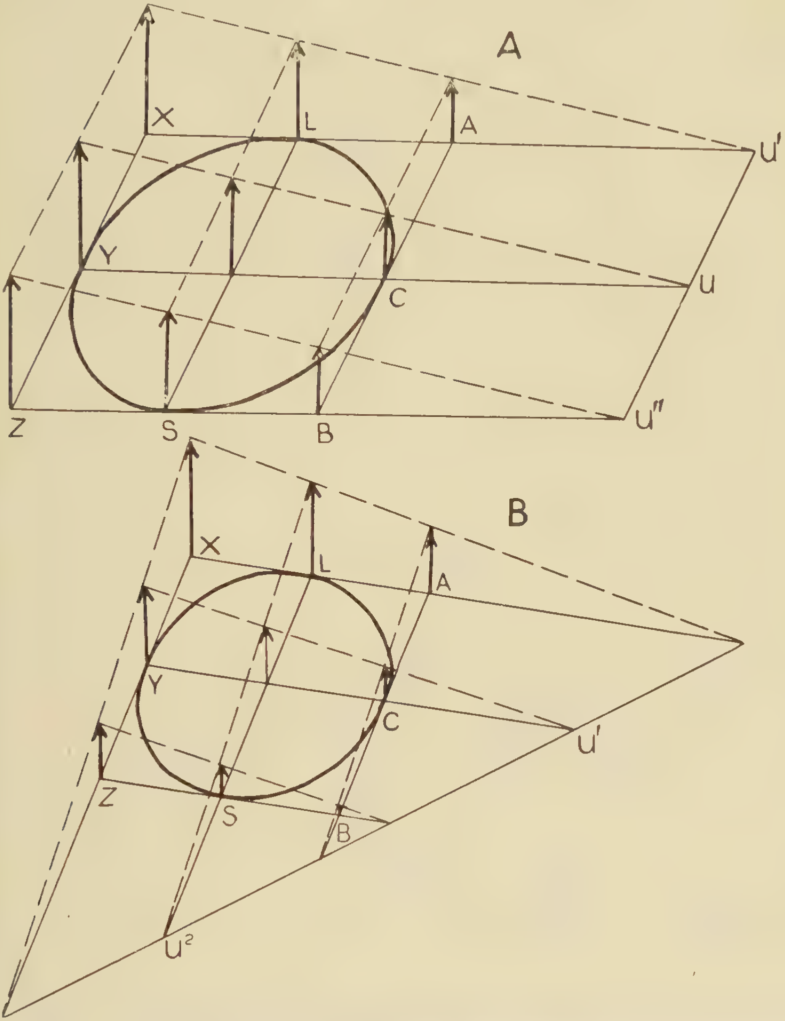


FIG. 6. Lison's interpretation of Huxley's hypothesis explaining the formation of: A, a plano-spiral shell; B, a turbinate-spiral shell. (After Lison, 1949.)

growth ratios (*CY* and *SL*) will result in the formation of a turbinate-spiral shell as postulated by Huxley (fig. 7, B represents the formation of a turbinate-spiral shell in gastropods).

DISCUSSION

The value of the spiral angle of the normal axis is dependent on the ratio of the radial and transverse components at the normal zone of the mantle edge

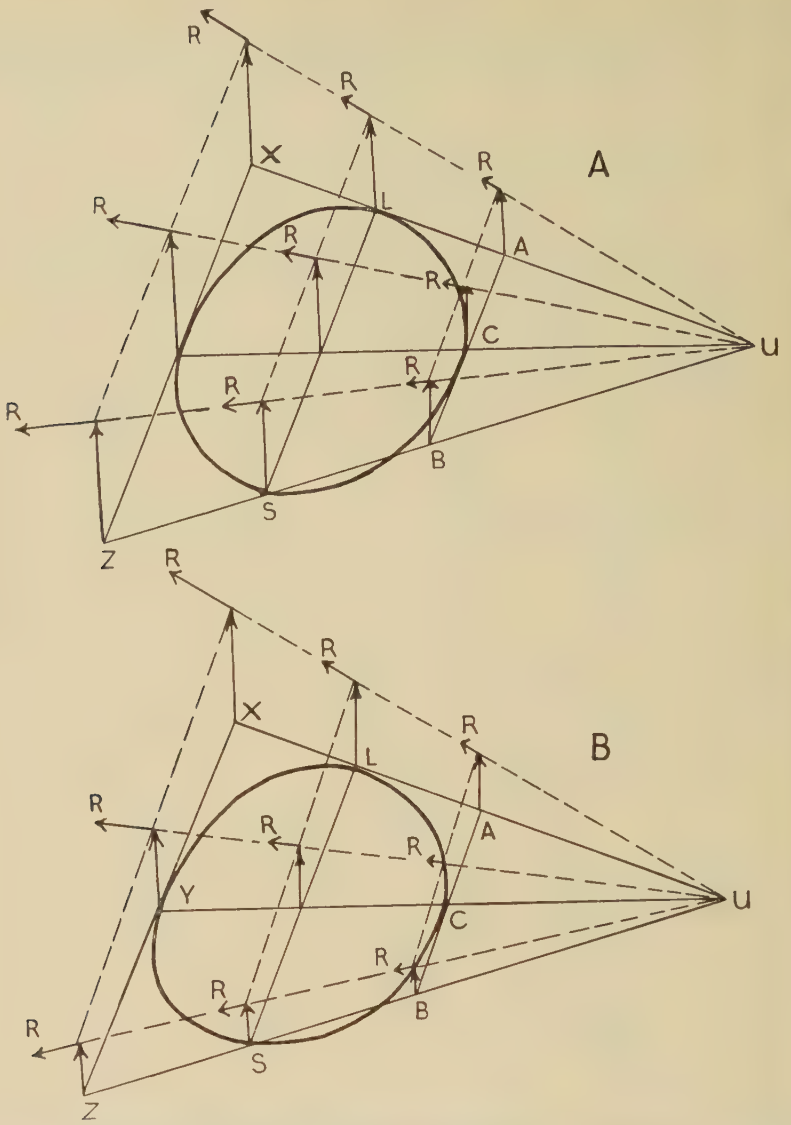


FIG. 7. A reconstruction of figs. 6, A and B, to show the radial component and the lines of growth radiating from a node of zero growth. A, a plano-spiral shell; B, a turbinate-spiral shell.

and is a measure of the concavity of the valves. The form (i.e. plano- or turbinate spiral) of the normal axis is dependent on the tangential component. In genera such as *Glycymeris*, *Tellina*, and *Ensis* where the form of the valves is not affected by a tangential component, the normal axis is a plano-spiral and the umbones oppose one another. In those genera in which the form of the valves is affected by a tangential component, e.g. *Arctica* and *Glossus*, the normal axis is a turbinate spiral and the umbones are directed anteriorly. The form of the turbinate spiral is governed by the values of the radial, transverse, and tangential components at the normal zone. The outline of the generating curve is dependent on the disposition of these components on each side of the normal axis.

Thus, the form of the shell valves in the Lamellibranchia may be considered with reference to: (i) the spiral angle of the normal axis, (ii) the form (i.e. plano- or turbinate spiral) of the normal axis, and (iii) the outline of the generating curve.

In the primitive bivalve, the form of the shell valves was probably the resultant of radial and transverse components symmetrically disposed on each side of a plano-spiral normal axis. Certainly this is largely the case in some modern genera, e.g. *Glycymeris* and *Tellina*. In genera such as *Anodonta* and *Ensis*, while the effect of the transverse component remains greatest ventral to the umbones and thus the normal axis is a plano-spiral, greatest marginal increase takes place posteriorly; the generating curve is asymmetrical about the normal axis. Nevertheless, the form of the valves in these genera is still the resultant of radial and transverse components alone. In many lamellibranchs, e.g. members of the Mytilidae, Veneridae, and Cyprinidae, the form of the valves is affected by an additional factor—the tangential component—and the normal axis is a turbinate spiral. In the majority of these genera, the radial and transverse components appear to retain more or less their 'primitive' symmetry on each side of the normal axis and, unlike *Anodonta* and *Ensis*, the inequilateral form of the shell valves is largely due to the tangential component.

Lison (1949) claims that the form of the shell, provided the generating curve is in one plane, is dependent on: (1) the angle of the directive spiral; (2) the angle of incidence, i.e. the angle between the 'directive plane' and the plane of the generating curve; (3) the outline of the generating curve. It will be seen that much depends on Lison's concept of the 'directive plane' of the valves. This is defined as follows:

> Chez les Lamellibranches existe toujours un plan et un seul, dans lequel s'inscrivent sur toute leur longueur une ou des côtes représentant des courbes planes. Ce plan, qui passe necéssairement par l'umbo, sera appelé le plan directeur de la coquille. Toutes les côtes situées de part et d'autre du plan directeur dessinent des courbes gauches, c'est-à-dire non contenues dans un seul plan.

According to Lison, the position of the 'directive plane' varies in different species of lamellibranchs and these variations exert a profound effect on the form of the shell. In *Pecten*, the 'directive plane' is perpendicular to the plane of the generating curve and the angle of incidence is 90°. In the majority of lamellibranchs, however, the 'directive plane' is more or less inclined to, and can almost coincide with, the plane of the generating curve.

In *Pecten*, *Glycymeris*, *Anodonta*, and *Ensis*, the plane of what is here described as the normal axis is identical with the 'directive plane' defined by Lison. But these are all species in which there is no tangential component affecting the form of the shell. In *Corbis fimbriata*, one of the examples quote by Lison as possessing the 'directive plane' inclined to the plane of th generating curve, there is a small tangential component. Thus, the norm axis is a turbinate spiral and does not coincide with the 'directive plane Fig. 8, A represents a shell in which the 'directive plane' is perpendicular to th plane of the generating curve (*AB*) and coincides with the normal axis; ther

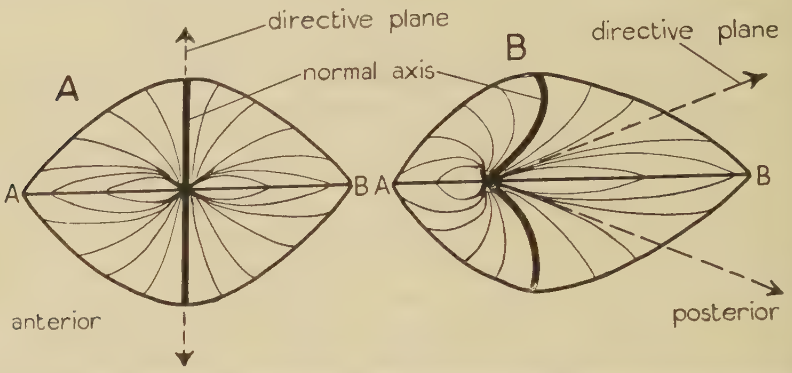


Fig. 8. Diagrams comparing the 'directive plane', as defined by Lison (1949), with the norm axis. A, a shell not affected by a tangential component; B, a shell affected by a tangenti component.

is no tangential component. This can be transformed into fig. 8, B by a tangen tial component affecting the form of the valves. The normal axis is no longe in one plane while the turbinate-spiral ribs posterior to the normal axis ar 'deflected' anteriorly. It follows that one of these ribs will come to lie effec tively in one plane. This change in the position of the 'directive plane' is th result of the tangential component. Moreover, in members of the Chamida and Glossidae (i.e. *Isocardia*), the magnitude of this tangential component i such that *all* growth curves are turbinate spirals; i.e. *there is no 'directive plane'*

I wish to express my gratitude to Professor C. M. Yonge for many sug gestions and invaluable criticism during the preparation of this paper and to Mr. R. B. Clark, of this Department, for assistance in many ways.

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A Histological Study of the Development of the Scales of the Largemouth Black Bass (*Micropterus salmoides*)

By MARY ALICE DIETRICH

(*From Simmons College, Boston, Massachusetts*)

With one plate (fig. 1)

SUMMARY

1. The scale method of age determination in largemouth black bass, *Micropterus almoides*, is valid at least up to and probably including the third year of the fish's life. A more careful study of older fish might reveal that it was valid beyond that point.

2. The annulus in a largemouth black bass is formed by a brief but gradual slowing down of growth and then its quick resumption. The annulus appears as a wide band on the anterior edge of the scale, and as a dark line (circulus) cutting across some inner dark lines (also circuli) on the lateral edges.

3. The scale is mesodermal in origin, and lies in a pocket surrounded by dermal tissue, which in turn is covered externally by a thin layer of epidermis.

4. The bony layer is formed by osteoblastic cells on its outer surface in a manner similar to the formation of dermal bone. Excess bony material is accumulated in ridges or circuli.

5. The fibrillary plate is formed by the fibroblastic cells on its inner surface.

INTRODUCTION

ALTHOUGH the method of determining age in teleost fish by counting rings is generally accepted as valid, work is still being done in the field in an attempt to accumulate further evidence bearing on the subject. It has been the purpose of this study to gain more information of a histological nature regarding the early development of scales. Largemouth black bass, *Micropterus salmoides salmoides* Lacépède (Bailey and Hubbs, 1949), was used in this work because of its particular importance as one of the leading game fish.

The scale of a largemouth black bass is of typical ctenoid type. It varies somewhat in shape in different parts of the body, but generally resembles that shown in fig. 1. The scale is considered to be mesodermal in origin, as it seems to be formed in a manner similar to that of dermal bone. It has a lobed anterior end, which is buried in the dermis of the skin, and a toothed posterior end, which is exposed, and overlaps the anterior parts of the next, more posterior scales. A thin layer of epidermis originally covers the dermal layer, but as the scale grows, the posterior edge moves backwards, and the epidermis will remain either as a thin coat over the exposed toothed part, or will be rubbed off (Hyman, 1947).

[Quarterly Journal of Microscopical Science, Vol. 94, part 1, pp. 71-82, March 1953.]

A general surface view of a scale will show the following parts: a *focus*, whi is a small clear area in the centre and presumably represents the original sca (Van Oosten, 1928); *circuli*, which are concentric (or nearly concentric) ridg around the focus; *radii*, which run from the region of the focus to the anteri margin; and *annuli*, which are zones of retarded growth (not shown in fig. 1 The annuli are so called because they are generally regarded as being form during the winter months when the growth of the fish is retarded because scarcity of food and low temperature of the water. This general assumptio however, varies not only among the authorities who study fish growth, b also from one species of fish to another. Thus, a careful study of annulu formation should be made for each species before using annuli as a certa indication of age.

In largemouth black bass, annulus-formation seems to vary from Apr (eleventh month) into July (fourteenth month), the majority being forme in April and May (Roach and Evans, 1948). Everhart (1948) found that i his work with the smallmouth black bass (*Micropterus dolomieu* Lacépède experimental annuli were formed in February and March. This unexplaine variation within a genus is one indication of the need of more specific studie both in the laboratory and in the field.

Historical Review

Concentric rings or zones in various parts have been used for a long tim as indicators of variation in growth caused by the changes in season (Thompson, 1948). In fish these rings have been observed in scales, otoliths, an bones, and are used not only as an indication of age, but also as a key to th growth of the animal itself, and thus to the environment in which it lived.

Some general observations of scales were made in the eighteenth and earl nineteenth centuries, but most of the specific studies correlating scales wit growth have been done in the last 50 years. The older work has been reviewe by Van Oosten (1928), and will not be discussed here.

Professor D'Arcy Thompson, in his book on *Growth and form* (1948), presents interesting information on the deposition of inorganic material in living organisms. He discusses the physico-chemical phenomenon of Liesegang's rings, which are periodic depositions from solutions. He offers this phenomenon as a possible explanation of the formation of 'age-rings', particularly in otoliths. He says further that although there is much supporting evidence for the scale method of age determination, that in light of the Liesegang phenomenon, we should not assume without doubt that these periodic rings or zones are accurate indicators of external conditions.

Before considering the histology of teleost scales, perhaps one further item should be noted in a general consideration of the validity of the scale method. The rate of growth in fish decreases with age (Lea, 1910), and consequently the older annuli are closer together, and often difficult to distinguish. Added to this is the possibility of various discrepancies, as suggested by Lee (1920), due to contraction of the previous year's layer, partial absorption of the scale

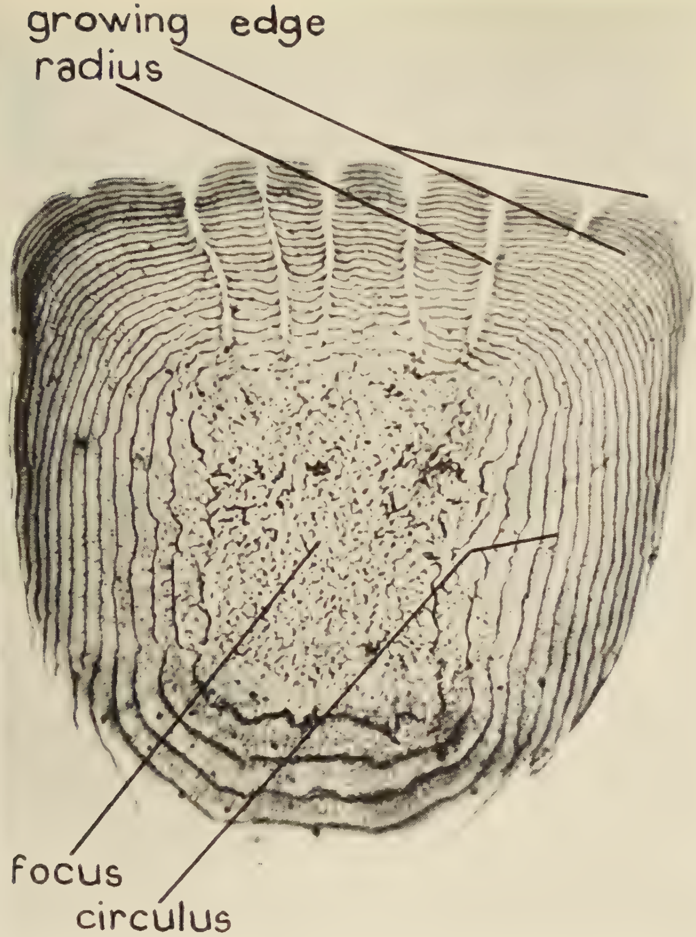

FIG. 1. Photograph of a whole mount of a scale at 9 months. Alizarin red S

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during spawning, and the formation of 'accessory' rings. All these possible variations serve further to emphasize the necessity for careful study of the growth of each species of fish and its scale before the scale method can be applied to it with any degree of certainty.

A detailed historical review of histological studies is given by Neave (1940), who indicates that the structure of a fish scale has been interpreted differently by different investigators. In general, the scale is considered to consist of a thin layer of bone, supported by one to many layers of material forming a fibrillary plate, and covered on both sides by one or more layers of cells. However, there has been much disagreement as to scale origin, the cause of the formation of the ridges and of the annuli on the scale, the order in which the various layers in the scale were deposited, and the function and consistency of the various parts. Because Neave's paper has been the major source of ideas for this work, I shall restate his conclusions briefly:

(1) The teleost scale is covered by two layers of cells on the external surface, and one layer internally; the latter is continuous with the deeper external layer.

(2) The intimate layers are osteoblasts and osteocytes, and are concerned with the formation of the bony layer.

(3) The fibrillary plate is laid down as collagen, but soon becomes infiltrated with an albumoid, ichthylepidin.

(4) The scale is mesodermal in tissue relationships.

(5) The ridges, formed between cell rows of the deeper external cell layer, are not products of specific cells, but depositions of excess bone-forming materials in the intercellular fluid not used by the growing margin.

(6) The radii represent lines of flexibility, as the bony layer is absent, and the underlying fibrillary plate lacks ichthylepidin.

MATERIAL AND METHODS

All the specimens for this investigation were furnished by the Ohio Division of Conservation, Section of Fish Management, under the supervision of Mr. Lee S. Roach. The material was collected at designated intervals, beginning with the day of hatching on 17 May 1949, and preserved in 10 per cent. formalin. Collections were made daily for the first 10 days, weekly for the next 6 samples, and then monthly. In November, the school was divided, and half was kept in an outdoor tank and half indoors. Thereafter, a monthly sample was taken from each half of the school.

Ideally, all the specimens used should have been from one school to reduce the individual variation factor. However, this was not possible, because after the January sample was taken, the tank in which the outdoor half of the school was located was flooded during some heavy rains, and lost. Subsequent outdoor representatives were taken from another school of the same age. A few checks were made also on scales collected from older fish in previous years.

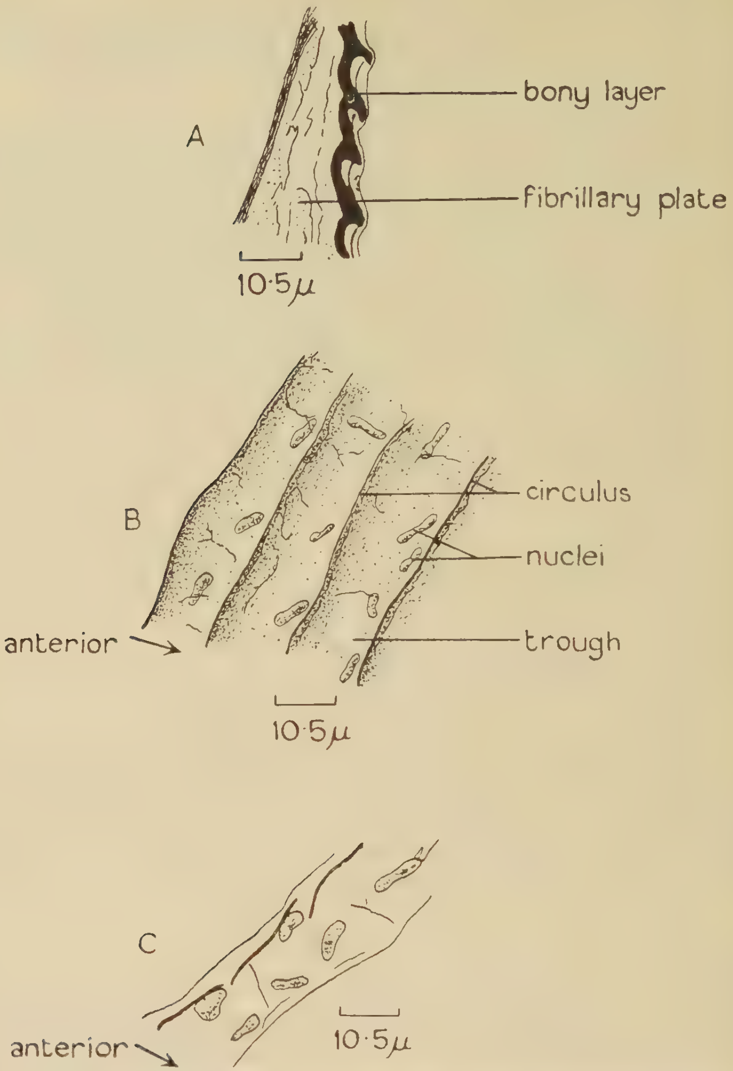


FIG. 2. A, detail of scale near anterior end. Composite drawing of sections of 9-month scal Alizarin red S and Heidenhain's iron haematoxylin. B, portion of outer surface. View of 11 month scale from outer side. Heidenhain's iron haematoxylin. C, same as B, but nearer anteri edge in growing margin.

The material was differentiated with alizarin red S in a method aft Williams (1946), and stored in xylene. Cross-sections were made by th celloidin-paraffin double imbedding method (Bensley and Bensley, 1938), an except for a few trial sections, were cut at 10μ. Thicker sections tended shatter too much to be of any value.

A number of stains were tried, but the most satisfactory were alizarin red Mallory's triple stain, Heidenhain's iron haematoxylin, and silver nitra

2 per cent.). Eosin was used occasionally as a counterstain for Heidenhain's ron haematoxylin, but the latter seemed clearer without a counterstain. The Feulgen technique was tried on sections of 10 μ and thicker, but discarded, or the time and care required in staining did not produce better results than ould be obtained with faster, easier stains. Mallory's phosphotungstic acid haematoxylin was tried in an attempt to differentiate cell outlines, but proved unsatisfactory. Delafield's haematoxylin was also used, but was not as good s some of the others. Permanent preparations were mounted in clarite.

OBSERVATIONS

Scales stained in alizarin red S and mounted in a flat or total mount give general overall pink colour, with a deeper pink in the ridges or circuli, the outer margins of the radii, and the growing margin of the scale (fig. 1). When he epidermis is still on the posterior, exposed part of the scale, this also ppears deeper pink. In a cross-section of a scale in this stain, the bony layer n the outer surface appears pink, and the fibrillary plate beneath it is colourless. Sometimes, if the light is just right, the furrows in the fibrillary plate, nd the outlines of cells between the ridges are faintly visible (fig. 2, A).

Scales were stained in Heidenhain's iron haematoxylin to show the nuclei. The circuli appear as dark lines around the scale at more or less regular ntervals, and are broken at the radii. Between the circuli on the outer surface re oblong nuclei and irregular, hair-like lines, which appear like cracks or canals between the nuclei and the ridges (fig. 2, B). This stain, like the alizarin red S, appears darkest at the circulus and fades out anteriorly across the trough until the next ridge. The nuclei of the outer cells are scattered unevenly across he scale, increasing in number towards the distal or growing edge of the scale. Between the circuli, or ridges, of the growing edge, the nuclei are more numerous (fig. 2, C), and in the radii and in the section between the last (newest) circulus and the edge of the scale are concentrated (fig. 3, A). The concentration around the edge of the scale is made greater because the cells re more than one layer thick. The nuclei in the radii and on the edge of the scale differ from those between the circuli in shape, because while the latter re all about the same oblong shape (considerably longer than wide), the former tend to be more rounded.

The nuclei of the lower surface are more of an oval or kidney-bean shape (fig. 3, B), and are apparently scattered over the surface in a thin double layer in no particular pattern. As one focuses the microscope from the inner to the outer surface, the cracks or canals previously mentioned as being between the nuclei and ridges are seen to be internal to the outer nuclei (fig. 3, C). Observations of the outer surface of a portion of a scale from which the cellular layer had been removed showed no cracks or canals, seemingly indicating that they had been removed, too.

The radii contain no bony material over the distal half, as indicated by the fact that they remain colourless in that portion when stained with alizarin red S. However, the borders of this part take a deep stain, as previously mentioned. The proximal half of the radii are the same shade of light pink the troughs between the circuli (fig. 1). The circuli, which do not cross radii, bend up a little into the radii away from the sulcus (figs. 1; 3, A). T nuclei on the distal edge of the radii in the troughs of the last four or five circ give an appearance of flowing into or out of the troughs.

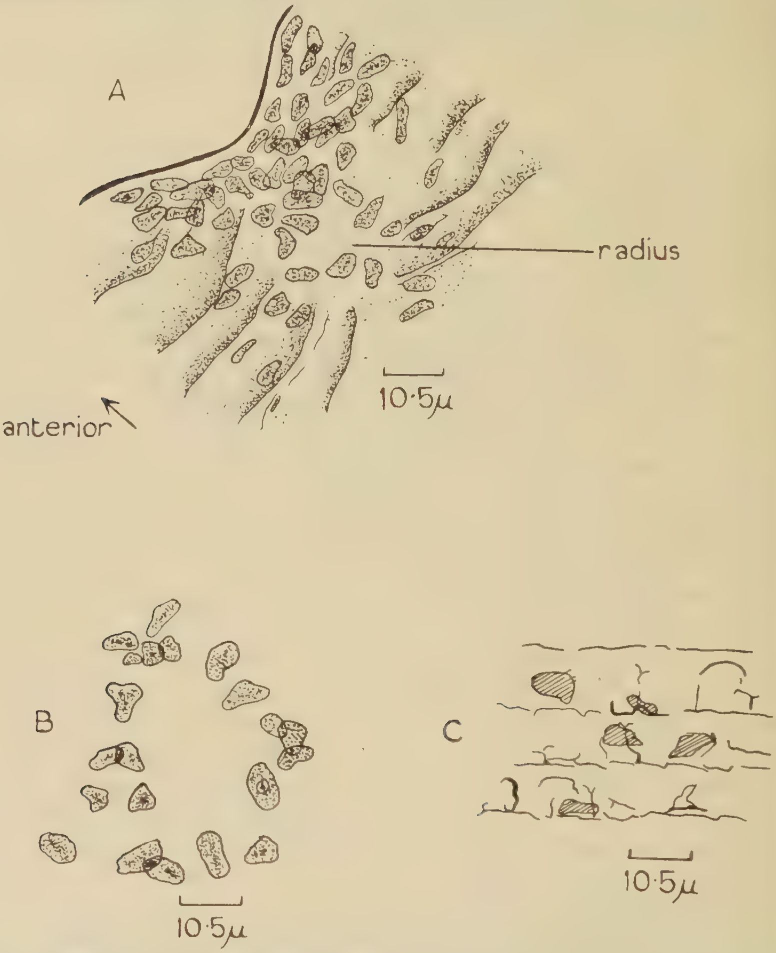


FIG. 3. A, detail of portion of growing margin, including part of radius. 11-month scal Heidenhain's iron haematoxylin. B, portion of inner surface. View of 1½-month scale fro inner side. Heidenhain's iron haematoxylin. C, same part of scale as B, but at a deeper focu

The circuli of young largemouth black bass are not difficult to distinguis but those of the older specimens are increasingly indistinct. On the anteri portion of the scale an annulus appears as a wide trough between two circul with the inner of the two circuli appearing more darkly stained. At the tw antero-lateral corners, the trough narrows, and the circulus bordering anteriorly bends round the corners sharply and cuts off three or more of th

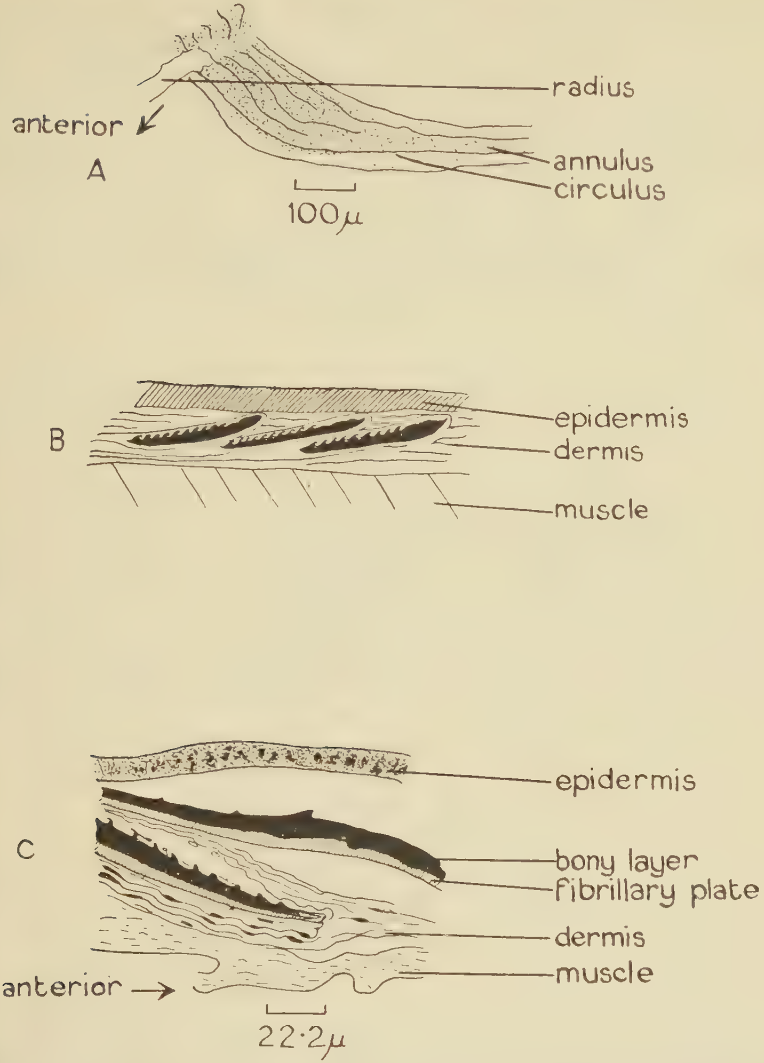


FIG. 4. A, portion of annulus on antero-lateral axis of scale. View of 15-month scale from outer side. Heidenhain's iron haematoxylin. B, diagrammatic section through skin to show scale and tissue relationships. C, transverse section through skin and scales of 1½-month fish, showing beginning of scale. Mallory's triple stain.

inner circuli, and then continues toward the posterior margin, paralleling the older circuli (fig. 4, A).

The scales of the largemouth black bass first appear when the fish is 1 month old. All sections made from that time up through the December samples, which were a little over 9 months in age, were of the skin and scales. From that time on only the scales were sectioned. Great care was taken to ensure that the sections studied had not been cut at such an oblique angle as to distort the true relationships of the various layers. A generalized diagram of a section has been made (fig. 4, B) to show these relationships, and at the sam time to indicate the general structure of the ridges. Those which are in th anterior part of the scale are smaller and closer together; those in the posteri part, fewer, larger, and farther apart—gradually ceasing to exist in the regio where the teeth would be located.

Part of a typical section is shown in a drawing made from a slide of a 44-da specimen (fig. 4, C). The drawing was made from a slide stained in Mallory triple stain observed at an approximate magnification of 430X. Details wer checked under magnification of 950X of this slide, and at both magnificatio of a similar slide stained in Heidenhain's iron haematoxylin. This sectio shows the origin of the scale in the dermis.

In a section stained in Mallory's triple stain, the bony layer of the sca appears dark blue, and the fibrillary plate is a yellow-red, becoming mor yellow towards its anterior edge. The dermis, from which the scales originat appears as a band of wavy striations, which is red with much blue scattere through it. The muscle under the dermis is orange, and the epidermis ove the scales has a lavender cast (light blue cytoplasm, and pink nuclei). Th part of the dermis which goes over the outer surface of the scale, and unde the next more anterior scale, loses its striated appearance after the fifth (mor or less) ridge, and becomes a narrow band of cells. The cells of the innermos layer are the same length and shape as the troughs between the ridges, an appear to have been pulled out from between the ridges during sectioning. I the section stained in Heidenhain's iron haematoxylin is not completely differentiated during the staining process, the older part of the fibrillary plate wil remain dark like the nuclei, while the newest anterior portion is colourless The cytoplasm and the bony layer do not take the stain.

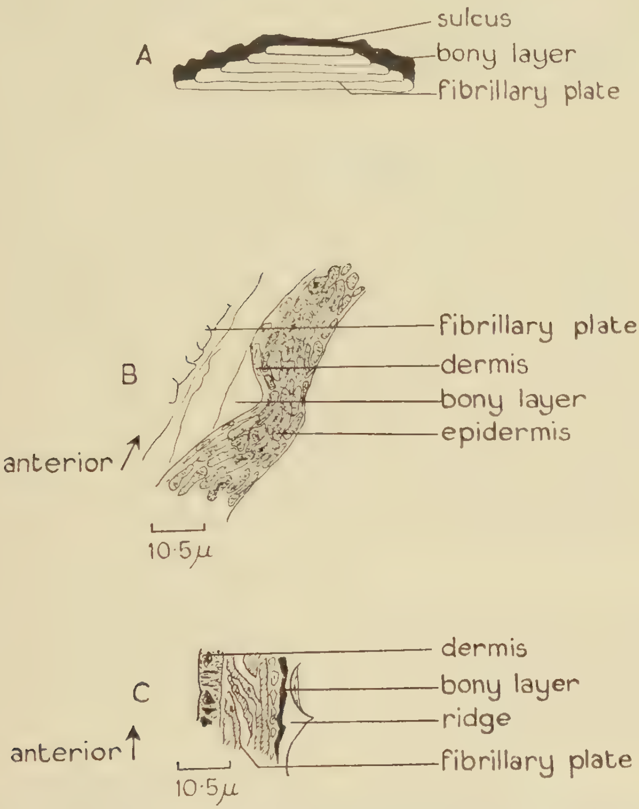
In the scales of older fish, the fibrillary plate is wider and in layers, but th bony layer remains the same in thickness. Fig. 5, A is a diagram to show th mode of growth of the fibrillary plate and the bony layer. Thus a scale i thickest through the region of the sulcus, and tapers toward the edges. Th fibrillary plate layers are orange-red, as in the younger scale section, whe stained with Mallory's triple stain, but each layer is separated from the nex by a fine line of material which is bright blue in colour (connective tissue blue)

The number of cells on the outer and inner surfaces is not easy to determine, at least with the stains used here. At the anterior growing margin, th cells are several layers thick (figs. 3, A; 4, C), but behind that on the oute surface, two layers of cells can be observed—one between the ridges, and on lying over those and the ridges (fig. 2, A). On the posterior part of the scal under the epidermis, no more than one layer of cells is visible. The latter ar small compared to the epidermal cells next to them (fig. 5, B). In a Mallory's triple stain these dorsal cells do not take a cytoplasmic stain, but show pin nuclei and faint cell outlines.

The cells of the inner surface are at least two layers in depth (fig. 3, B), an probably more (fig. 5, C). They are very thin, and appear in cross-sectio somewhat like the epidermis layer.

Scales from 12- and 13-month samples reveal the annulus formation. On e earlier scale, the annulus is starting to form on the anterior edge; on the ter one, it is still incomplete on the lateral edges.

About the only comparison which can be made between the scales of the sh kept in the indoor tank, and those kept outdoors, is that the annuli on the



IG. 5. A, diagrammatic section through a scale to show mode of growth. B, detail of 10-month cale near posterior end (transverse section). Heidenhain's iron haematoxylin. C, detail of 9-month scale near anterior end (transverse section). Heidenhain's iron haematoxylin.

cales of the indoor samples appear no sooner than those of the outdoor amples. An attempt to compare size and growth of fish and scales did not eem feasible here because the school was broken, and because only a few pecimens were collected for samples. In comparing the two histologically, no lifferences are noticeable.

DISCUSSION

The scale of the largemouth black bass is mesodermal in origin, for it grows ut of and is surrounded by dermis. Over this layer of dermis and scales rows a thin layer of epidermis, which is somewhat stratified in structure. A scale is composed of a dorsal bony layer of even thickness, and a ventr fibrillary plate, which increases in thickness as the fish grows.

The bony layer of the scale is formed like dermal bone by the cells whi are concentrated around the growing margin of the scale. These cells a probably comparable to the osteoblast cells forming dermal bone, but th differ in that their cytoplasm is used up, and only a long narrow nucleus an a cell membrane remains between the ridges behind the growing margin. Or other striking difference between scale formation and dermal bone formatio which may have some bearing on the statement just made, is that mo material is secreted than is used by the growing edge of the scale. This exce material is then piled up and forms a circulus or ridge. The irregular lig lines observed around these dorsal cells and between the ridges are probab the remains of cytoplasmic processes which normally connect osteoblast With regard to the double layer of cells on the anterior outer surface, it possible that one layer of cells does not furnish enough bony material, but th the second layer contributes more than enough. The lower, more intimat layer, because it is closest, contributes the major part of the material to th formation of the bony layer; the outer layer contributes only at those poin where the ridges touch it. Thus the cells of the outer layer are slightly large

The osteoblasts originate in the dermis at the point of origin of the scal and then apparently multiply on the edge in the growing margin as neede There also seems to be some migration of cells and bony material primaril within the growing margin from out of the troughs into the radii and ou towards the edge of the scale. Perhaps some of the surplus bony material alon the side of the radius is used to cover over the radius when calcification ha been completed and the radius is no longer needed for the flow of materials Perhaps the radii do aid in the flexibility of the fish (Neave, 1940), but tha purpose seems incidental to the more important function as a 'canal'. Th flexibility theory does not explain the accumulation of cells within and bon material beside each radius.

The fibrillary plate is also laid down by cells which originate from th dermal pocket surrounding the young scale. As Neave (1940) suggests, th fibrillary plate is first laid down as collagen (it appears yellow-orange in sectio stained with Mallory's triple stain, and colourless in Heidenhain's iron haema toxylin), and soon becomes infiltrated with ichthylepidin (giving the layer red-yellow cast in Mallory's triple stain and remaining dark in Heidenhain' iron haematoxylin). Each layer in the fibrillary plate is bordered with a thin layer of connective tissue, which probably makes the whole plate less rigid.

The cells of the inner surface vary from a depth of two and probably more cells in the anterior region to a single layer posteriorly. These cells could probably be called fibroblasts, for their nuclei resemble those o common connective tissue cells.

The layers of the inner and outer cells are continuous round the anterior margin of the scale, thus enclosing the scale in a pocket of dermal cells, which, although structurally alike, have become differentiated in function. The thick ayer of cells, although greatest on the anterior margin, is also visible on the ateral edges of the scale. The author therefore believes that once the original cale has formed, further cellular material is not derived from the dermis, but s furnished completely from the growing edge. No dividing cells were bserved, however, to substantiate this hypothesis.

The date of the annulus formation, and the fact that no difference was bserved in the mode and time of formation between scales from fish kept utdoors at a lower temperature and those kept indoors at higher temperatures, ould lead the author to conclude that the slowing down of growth was conrolled by factors within the fish.

The slowing down of activity in the growing margin is shown by the ridges, hich are not as high (in a total mount stained in alizarin, they are lighter in olour), or complete on the lateral margins. Less material was in excess to form hese circuli. On the anterior edge, where most growth occurs, the troughs orming the annulus are about twice the width of the troughs between the receding and ensuing circuli. The assumption is that the first half of the rough is formed during the slow growth period, and the second half after ormal growth is resumed. The latter half is then followed by its normal irculus, which forms around the anterior and lateral edges parallel to them. his description might furnish a reason for the difficulty in distinguishing nnuli of fish 3 or 4 years of age and older. As a fish matures, its relative growth ate decreases. This would place the annuli closer together, and probably educe the wide trough, making the entire area appear very irregular and ndistinguishable, or at least uncertain.

I would like to express my appreciation to Dr. Robert S. McEwen and r. Hope Hibbard of Oberlin College for their professional and personal nspiration in the development of this work. Likewise, I would like to thank r. Lee S. Roach of the Ohio Division of Conservation and Natural Reources, who not only brought the problem to my attention, but who also urnished the many samples of fish.

The work was done as a partial fulfilment of the requirements for the egree of Master of Arts in the Department of Zoology, Oberlin College, berlin, Ohio.

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The Structure of the Cornea as Revealed by Polarized Light

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SUMMARY

Studies of corneal sections in polarized light indicate that there are about 100 mellae in the cat cornea; the coefficient of fibre birefringence is 0·0030.

No evidence was found of preferential orientation of fibre directions. Corneal disks amined by an immersion technique show that the cornea exhibits both form and trinsic birefringence of positive sign with a micellar refractive index of 1·50–1·52. ne values of both form and intrinsic birefringence in various fixatives can be ex-ained on the basis of swelling, which is both inter- and intra-micellar. The micellar lume according to the Wiener relationships does not exceed 3 per cent.; possible uses of this are discussed.

MEASUREMENTS of the retardation of the cat cornea as a whole (Stanworth and Naylor, 1950) can be analysed mathematically (Stanworth d Naylor, 1952) and indicate that the coefficient of birefringence of the con-tuent fibres of the cornea is about 0·0028. In order to investigate this value perimentally, antero-posterior sections 15 μ thick were prepared from fresh t corneae by freezing methods or from formalin-fixed corneae by prior em-dding in paraffin wax. When these were examined under the polarizing icroscope it was found that the retardation varied considerably according the method of preparation and the mounting medium employed, indicating at the corneal birefringence is in great part due to the form or textural riety. The investigation was therefore extended and approached from two pects, the microscopic and the sub-microscopic structure.

icroscopic structure

Frozen sections 15 to 25 μ thick were prepared from fresh corneae and unted in Ringer–Locke solution on glass slides. A polarizing microscope s constructed on a horizontal optical bench and the slides were held in a unting which permitted sagittal, lateral, and vertical linear movements as ll as rotational movements about horizontal and vertical axes. Measure-nts of retardation were taken by means of a quarter-wave plate compensator ing a parallel beam of monochromatic light (5461 Å) from a mercury arc. ne readings varied from zero on those fibres lying perpendicular to the plane the section to a maximum on those fibres lying parallel to the plane of the tion. The thickness of the section being known, the latter figure enables coefficient of birefringence of the fibres to be calculated directly.

uarterly Journal of Microscopical Science, Vol. 94, part 1, pp. 83–88, March 1953.]

It is a curious and well-known fact that all antero-posterior sections of t cornea, in whatever plane they may be made, always have the appearance being made up of fibres cut lengthways, and this has always been attributed their extreme transparency and optical homogeneity. Since the use of pola ized light accentuates differences in intensity transmitted by different fibr it renders their boundaries more easily discernible. For any one analys position, this is only the case for a certain number of the boundaries, but was found that by rotation of the analyser a position could be found to ma almost any boundary visible. A count of individual zones of optical di continuity can thus be made; in the cat cornea of thickness 0·5 mm. in t centre, there are apparently about 100 distinct zones, in place of the 40 whi are visible by ordinary illumination. The figure of 60 layers in the hum cornea (Bowman, 1847) is thus probably also an underestimate.

In the literature some confusion exists in the terminology of the constitue elements of the corneal stroma and it might clarify the situation somewhat these zones were referred to as lamellae, i.e. aggregates of parallel bundles fibres into thin sheets one fibre thick; in the case of the cat, therefore, t thickness of these is about 5μ. The most consistent readings were obtain from sections 15 to 25μ thick mounted in Ringer-Locke solution; these ga a value of 0·0030 for the coefficient of birefringence of the fibres, a figu which is in good agreement with the theoretical predictions. Similar resu were obtained with sections varying from 10 to 30μ in thickness, but thick sections, apart from being more difficult to measure, gave much lower valu This is due to the fact that unless such sections are cut exactly through t corneal vertex, the fibres of one layer overlap those lying more superficial more deep; the retardation of one layer is generally in the opposite sense the next and the effective birefringence is thus lowered.

It might be expected that readings of the retardation produced by each fib would enable the angle between the direction of light passage through the fib and its optic axis to be calculated. This angle is the complement of the ang at which the fibre lies to the plane of the section. In this way, the suggesti of Kokott (1938) that there is a preferential orientation of the corneal fibr would be investigated. There are many practical difficulties, however. T analyser displacement for extinction when using a quarter-wave plate compensator does not exceed about 10°, and as no half-shade device can be us on account of the inhomogeneity of the field, the accuracy of setting is n great. Köhler compensators are unsuitable since their accuracy decreases the difference in retardation of the specimen and compensator increases an we are dealing here with retardations varying from zero to a maximum Another unfortunate drawback is the ambiguity inherent in the mathematic calculations so that a fibre tilted at an angle, say, β, to the plane of the se tion produces exactly the same retardation as one lying at $(180-\beta)$. In theor tilting the slide about an axis perpendicular to the fibre direction should enab one to resolve this problem by observing the resultant rise or fall in retardati as the direction of light passage approaches or recedes from the optic axis e fibre. In practice the technique was found to be extraordinarily difficult; ther fibres tend to overlie the observed fibre and hence to influence the tardation if the tilt is excessive; it is difficult to keep the point of observation ationary in all planes; and with such a low birefringence, the effect is generlly not greater than the margin of error. In view of this and the considerable emands made on the visual capacity of the observer this attempt was abanoned and observations were confined to readings taken with the incident ght normal to the plane of the section. Although the ambiguity thus remains, e readings can nevertheless be analysed in such a way as to detect any referential orientation of fibres in the plane of the section.

A large number of sections cut in various meridians was examined and up 65 individual measurements were made on each. Analysis showed no evience of preferential orientations of fibres at different depths in the cornea.

ub-microscopic structure

The only studies of corneal ultrastructure available in the literature appear be those of Hertel (1933), who used X-ray diffraction methods, and the rief investigation by means of the electron microscope made by Sebruyns 950). The primary difficulty in applying the well-known immersion techique is the statistically isotropic appearance of the cornea for normal light cidence. This was overcome by taking measurements of retardation with ght passing obliquely through pieces of cornea of thickness *d* and calculating e birefringence ω from the following formula:

$$\omega = \frac{\theta . \lambda}{\pi . d . \sin\alpha \tan\alpha}$$

here λ is the wavelength (5461 Å) employed, θ is the analyser shift from the ormal crossed position to the position of extinction, and α is the angle beveen the optic axis and the direction of light passage.

METHOD

Circular disks $6\frac{1}{2}$ mm. in diameter were removed from cat corneae by means f a punch and were supported between glass plates in a rotatable mount. his was placed on the axis of a horizontal polariscope, fitted with a quarterave plate compensator, so that in the zero position, monochromatic (5461 Å), arallel light from a mercury arc fell normally on the corneal disk. The material as obtained from cats used for other experiments, the eyes being enucleated mmediately after the induction of anaesthesia and immersed in one of four uids; namely, $2\frac{1}{2}$ per cent. formal saline, 35 per cent. alcohol, 5 per cent. ercuric chloride, and Ringer–Locke solution. Those placed in the Ringer–ocke solution had the cornea removed and disks trephined from them in less an 3 hours, so that the latter will be referred to henceforward as 'fresh disks'. isks from those immersed in preservative fluids were cut after periods rangg from 2 days to 3 months and are termed respectively, formalin-fixed, lcohol-fixed, and mercury-fixed disks.

In general, the thickness was measured by a micrometer, the disk immers in methylated spirits for several hours, transferred to absolute alcohol and l overnight. Thickness and retardation were measured the following morni and the disk transferred to the immersion medium for a period of 24 hou this procedure was repeated for six media of different refractive indices. T immersion fluids employed included ethyl and butyl alcohol, chlorofor glycerine and water mixtures, benzene, xylene, aniline, and mixtures of xy and methylene iodide.

RESULTS AND DISCUSSION

For each type of disk birefringence ω was plotted against refractive ind n_2. A series of theoretical curves was calculated from the following approxim tion of the Wiener formula (Bear, Schmitt, and Young, 1937):

$$\omega_{\text{form}} = \frac{d_1 d_2 (n_1^2 - n_2^2)^2}{2[(d_1 + 1)n_2^2 + d_2 n_1^2](d_1 n_1 + d_2 n_2)}$$

where d_1, d_2 are the respective volumes of particles and surrounding mediu and n_1, n_2 are the respective refractive indices; and the most suitable cur was selected (fig. 1).

The majority of points fell very near to the theoretical curve but seve anomalous results were obtained. In aniline the birefringence was found be negative and in chloroform it was unduly high. The first of these pec liarities is mentioned by Chinn and Schmitt (1937) and was considered to due to orientated embedding, but there seems to be no report of analogous ha penings with chloroform. Peculiarly low values of birefringence (about 1·50 10^{-4}) were found in glycerine. No significant differences were observed wi this substance between fresh and fixed disks, but difficulties were encounter with fresh disks in most immersion fluids on account of the tendency to rap opacification and curling and wrinkling of the tissue. These led to inaccur cies in readings both of thickness and retardation and probably explain th anomalous slope of curve *B* in fig. 1.

The conclusion is, generally, that the total birefringence in the cornea co sists of both intrinsic and form birefringence, the sign of both being positiv Since the former variety is due to molecular asymmetry, this indicates th the molecules have their long axis in the fibre direction. In preparations whic differ only in their degree of swelling, assuming no change in the orientatio or in the birefringence of the molecule itself, the intrinsic birefringence shoul be inversely proportional to the volume. Practically all the corneal swellin is at right angles to the plane of the fibres, and therefore the product of in trinsic birefringence and thickness should be approximately constant. Th figures for formalin, mercury, and alcohol-fixed disks are $2{\cdot}68 \times 10^{-4}$, 2·33 10^{-4}, and $2{\cdot}60 \times 10^{-4}$ and it may be deduced, therefore, that swelling and fixa tion bring about no gross disorientation of the molecules.

It is of interest also to examine the relations between thickness and bire fringence for a particular method of fixation and treatment but the prese range of thickness obtained was not, in general, sufficient to enable this to be done satisfactorily. An exception may be made in the case of the 15 alcohol-fixed disks, which have variations in thickness from 0·76 mm. to 1·33 mm. and can be divided into 4 groups, namely: 1 of 3 disks with mean thickness 0·83

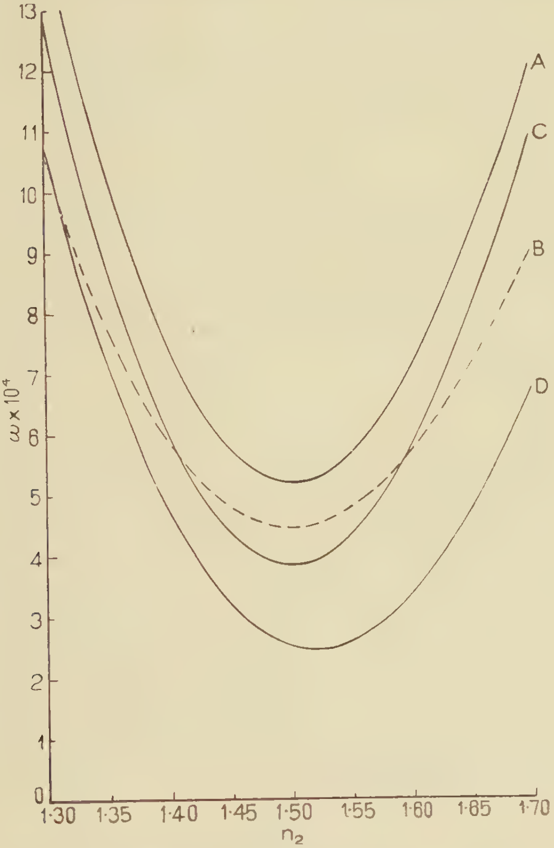

FIG. 1. Variation of birefringence (ω) with refractive index (n_2) of immersion medium in corneal disks from fresh and fixed eyes. A, formalin: B, 'Fresh': C, mercury: D, alcohol.

and 3 of 4 disks each, with mean thicknesses of 0·95 mm., 1·12 mm., and 1·27 mm. The respective values for the products of intrinsic birefringence and thickness are $2{\cdot}80 \times 10^{-4}$, $2{\cdot}56 \times 10^{-4}$, $2{\cdot}72 \times 10^{-4}$, and $2{\cdot}22 \times 10^{-4}$. No great accuracy is to be expected on account of the relatively small number of readings available, but the difference between each group does not suggest undue disorientation of the molecules.

Form birefringence is due to the asymmetry of molecular aggregates or micelles and its positive sign indicates that the corneal micelles are of the rodlet type. The minima of the curves in fig. 1 show that the refractive index of the micelles under these conditions lie in the region of 1·50–1·52. The value of $d_1 \times d_2$ in the Wiener formula is generally less than 0·03 and since the protein content of the cat cornea is about 25 per cent. (Duane, 1949), we can deduce either that the corneal micelles differ greatly in form from the geometrical rods considered by Wiener, or that only 3 per cent. of the total volume of the cornea is in the form of rod-like micelles. A third and likely alternative which forms the most profitable basis for discussion is some combination of the two former. Among the factors which would lead to a low value of $d_1 \times d_2$ we can list the following: (1) the major portion of the corneal protein may lie in molecular chains, not in micelles; (2) the micelles may be of the fringed variety, the boundaries being much less sharp than in the ideal mixed body considered by Wiener; (3) the micelles may not lie parallel to the long axis of the fibre; some support for this supposition comes from Sebruyns (1950), who, by the use of the electron microscope, found a helicoidal arrangement of fibres; (4) the low value of $d_1 \times d_2$ could be due either to a large number of very small or to a smaller number of larger particles. Polarization studies cannot distinguish between these two types, which are, of course, still small with respect to the wave-length of light.

To return to the experimental results; despite the limitations of the method, it might be expected that there would be a regular inverse relationship between the volume product $d_1 \times d_2$ and the thickness if the micelles remain unchanged during swelling; the swelling being, in fact, inter-micellar. The product of d_1, d_2 and thickness should be constant under these conditions. In fact the respective values for formalin, mercury, and alcohol-fixed disks are 0·0156, 0·0180, and 0·0239, which, since the mean thicknesses of these disks are 0·52, 0·60, and 1·06, suggests that the size of the micelle increases with swelling. That is, some of the swelling is intra-micellar. This observation agrees with the findings from X-ray diffraction studies of Kuntzel and Prakke (1933), for collagen and Hertel (1933), for the cornea. The above figures indicate that for an increase of 100 per cent. in thickness the micelles swell by about 50 per cent. in the fixed cornea.

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Formalin-Sensitive Cells in Spinal Ganglia

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With two plates (figs. 1 and 2)

SUMMARY

Spinal ganglia of rabbits and cats were prepared for histological examination in a riety of ways. Dark staining multangular cells are present in sections after fixation ith formaldehyde succeeded by (*a*) alcoholic dehydration; (*b*) sectioning with the eezing microtome. Sections treated in other ways, including freezing-drying, only ve the typical small and large round cells. It is suggested that certain groups of lls, sensitized by formaldehyde fixation, can react by shrinkage on subsequent treat- ent by alcoholic dehydration or other procedures.

FROM time to time it has been contested that there are different nerve cell types in the spinal ganglia and that, while the typical pseudo- nipolar cell dominates the cell population, it does not altogether monopolize Amongst the first to study the cells of spinal ganglia were Retzius (1894), ogiel (1896), Lenhossék (1896), Ramón y Cajal (1906), Marinesco (1906), ageotte (1907), and Marinesco and Minea (1908). Using different histologi- l methods, they all commented on the occasional finding of nerve cells sembling motor cells. Kiss (1932) recorded the presence of a significant umber of dark-staining 'multipolar' cells in the ganglia of spinal and cranial erves after formalin fixation and prolonged treatment with osmium tetroxide. n the basis of their appearance, he suggested that they were visceral efferent lls. Blair, Bacsich, and Davies (1935) confirmed the presence of these aracteristic cells in spinal ganglia fixed in formalin. They could, however, d no evidence of multiple cell processes. They therefore preferred to scribe them as 'multangular' rather than multipolar cells, and concluded at any assessment of function would depend on experimental evidence.

The present work is an account of the effect of various methods of fixation, cluding freezing-drying, on the cytology of spinal ganglia, with special ference to alterations in shape and staining reaction.

MATERIAL AND METHODS

The material consists of spinal ganglia from rabbits and cats (young adults). he animals were killed by dislocation of the neck and the spinal ganglia pidly dissected out. The ganglia were treated as follows:

Group A, fixed in 4 per cent. aqueous formaldehyde-saline solution, de- drated and embedded in celloidin-paraffin.

uarterly Journal of Microscopical Science, Vol. 94, part 1, pp. 89–92, March 1953.]

Group B, frozen by placing in isopentane immersed in liquid nitrog dried in a vacuum for 48 hours at −35° C., and embedded directly paraffin.

Group C, fixed in 4 per cent. aqueous formaldehyde-saline solution a sectioned with the freezing microtome.

Group D, fixed in 4 per cent. aqueous formaldehyde-saline solution, teas out and examined under the microscope (i) unstained, and (ii) after staini with 0·5 per cent. aqueous toluidine blue.

Group E, fixed in 4 per cent. aqueous formaldehyde-saline solution, th treated as in group B.

Group F, fixed in Bouin's solution, alcohol/acetic acid/formalin, Zenk formaldehyde, or osmium tetroxide, dehydrated and embedded in celloid paraffin. The osmium-fixed material required no further staining. The oth sections were stained with 0·5 aqueous toluidine blue or haemalum and eos or by silver impregnations.

DESCRIPTION

Group A shows the small or large typical round cells with their enclosi nucleated capsule. In addition, a conspicuous feature of the sections is t dark-staining multangular cells, which may constitute as much as 10 p cent. of the total number of cells present in any one section (fig. 1, A).

Group B. In these sections (fig. 1, B), the cells are larger and much mo openly spaced throughout the section. There are no dark multangular ce and they are all typical large or small round ganglion cells. In sections stain with toluidine blue, the Nissl material is present in characteristic granul form. Here and there amongst the larger cells of the spinal ganglia of t rabbit are one or two with particularly coarse granules in the cytoplasm.

Group C. Sections are similar to those in group A and show the da multangular cells in addition to the small and large round cells (fig. 1, C).

Group D. No dark multangular cells can be seen in the teased gangli either unstained or after staining (figs. 1, D and E).

FIG. 1. Photomicrographs of spinal ganglia of rabbits (young adults).

A, group A (fixed in 4 per cent. formaldehyde-saline, embedded in celloidin-paraffin aft alcoholic dehydration; toluidine blue staining). Observe large and small pale cells and t numerous dark stained multangular cells. × 230.

B, group B (frozen-dried; paraffin embedded; toluidine blue staining). Only small an large pale cells can be seen. There is a complete absence of the dark multangular cel Though the magnification used was the same, all cells are noticeably larger than in th previous specimen. There is also less shrinkage of the interstitial connective tissue. × 23

C, group C (fixed in 4 per cent. formaldehyde-saline; cut by freezing microtome; toluidi blue staining). Apart from the small and large pale cells, the dark multangular cells are a obvious feature of this specimen. ×285.

D, group D (i) (fixed in 4 per cent. formaldehyde-saline; teased preparation; examined an photographed by phase-contrast microscopy). Dark multangular cells are completely absen ×250.

E, group D (ii) (fixed in 4 per cent. formaldehyde-saline; teased preparation; toluidine blu staining). Dark multangular cells are completely absent. ×250.

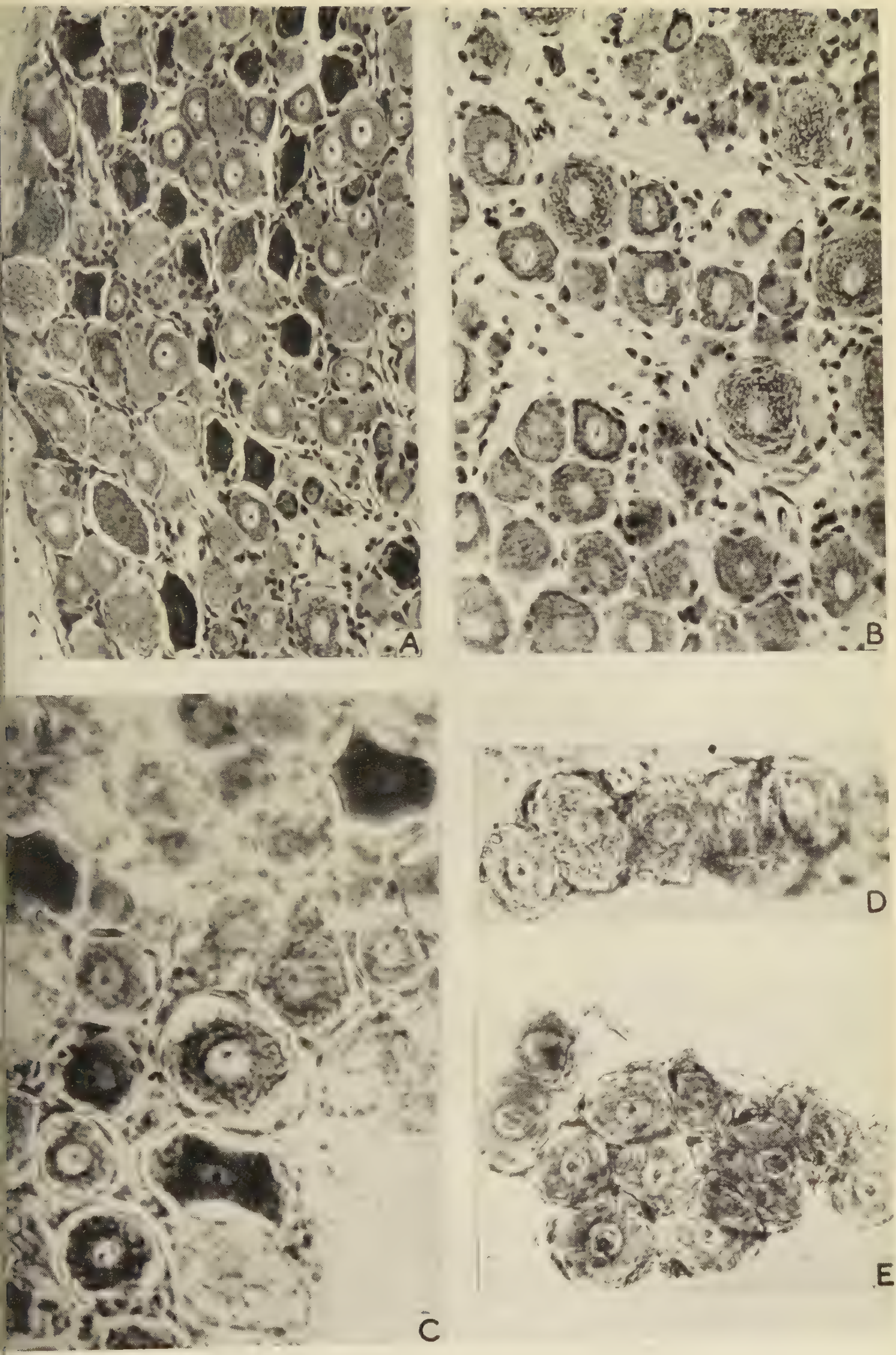

Fig. 1

P. BACSICH and G. M. WYBURN

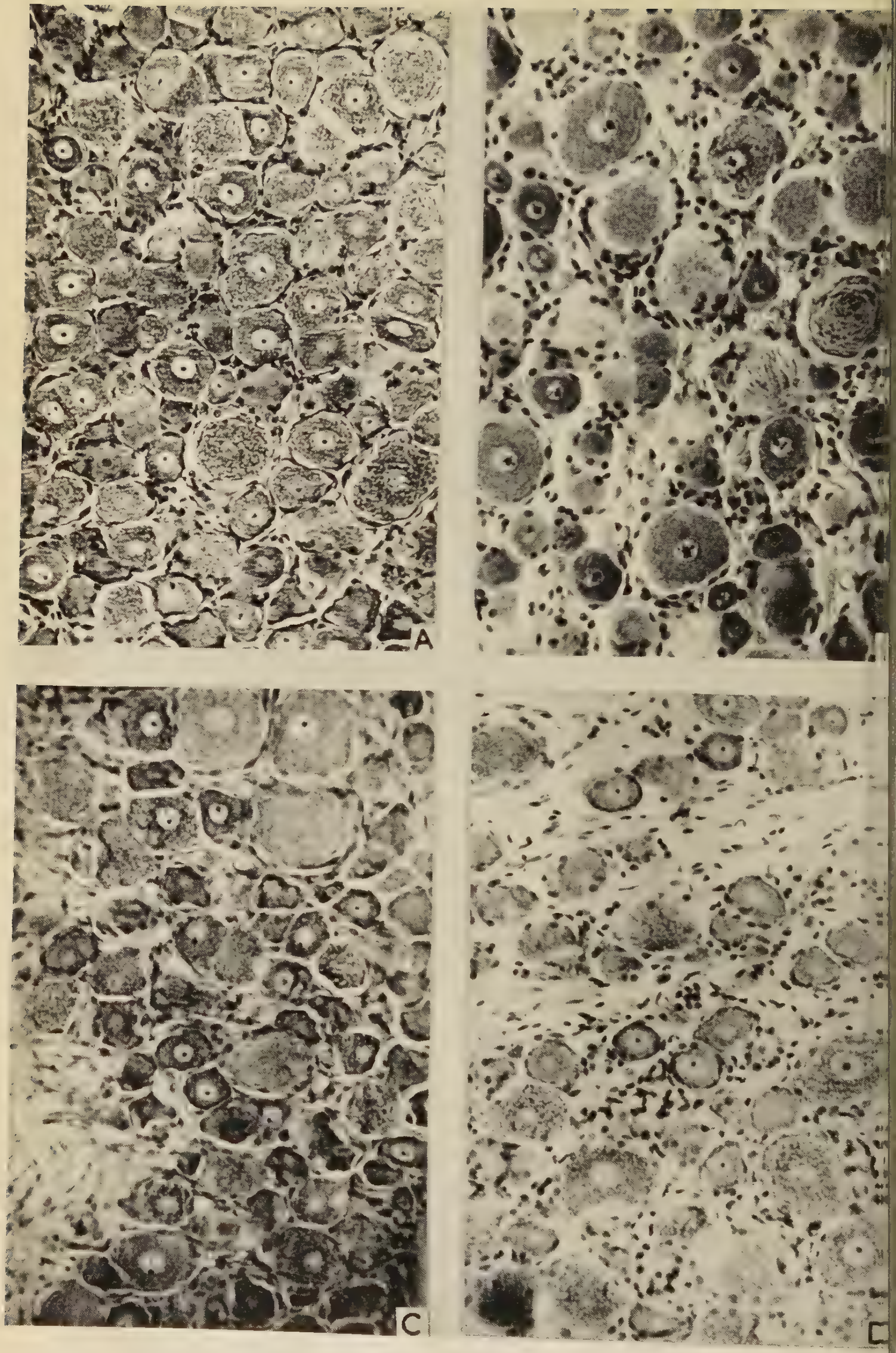

Fig. 2
P. BACSICH and G. M. WYBURN

Group E. There are no dark staining multangular cells (fig. 2, A). The round cells are smaller than in group B.

Group F. There are small and large round cells, but no multangular dark staining cells (figs. 2, B–D).

DISCUSSION

If it can be assumed that freezing-drying leaves the tissues structurally in their natural state, then dark multangular cells are not present as such during life. This particular type of cell only occurs in sections of spinal ganglia which have been fixed in formalin, and they are not found after fixation with other chemical reagents, even when these contain formalin, e.g. Bouin's solution, Zenker-formaldehyde, or alcohol/acetic acid/formalin. Formaldehyde fixation by itself does not determine the appearance of the dark multangular cells as they are not present in sections of spinal ganglia which have been fixed in formaldehyde, teased out and examined without further preparation (group D), or when formalin fixation was followed by freezing-drying (group E). The causal mechanism is therefore not a simple one but a combination of factors. Formalin fixation with alcoholic dehydration is effective in this respect (group A). The dark multangular cells can also be produced after formalin fixation when there is no alcoholic dehydration, e.g. in group C, when the formalin-fixed ganglia were cut with the freezing microtome. Alcoholic dehydration is therefore not an essential component of the causal mechanism, which apparently consists of a specific factor—formalin fixation—in combination with any one of several procedures, such as alcoholic dehydration or slow freezing. It is suggested that formalin sensitizes a particular group of cells so that they react to the appropriate secondary factor by shrinkage. The shrinkage would be responsible for the characteristic multangular shape and also the concentration of Nissl material resulting in dark staining.

Formalin, first used as a fixative by Blum (1893), was recognized by Van Gieson (1895) and Gerota (1895) as a particularly useful fixative for nervous tissues. Lubarsch (1895) complained that formalin produced too much shrinkage, while Parker and Floyd (1895) and Marina (1897), on the other hand, observed swelling of the cells of the central nervous system after formalin fixation. These divergent observations were finally reconciled by Tellyesniczky (1926), who affirmed that the immediate effect of formalin fixation

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FIG. 2. Photomicrographs of spinal ganglia of rabbits (young adults).

A, group E (fixed in 4 per cent. formaldehyde-saline, frozen-dried and paraffin embedded; toluidine blue staining). Dark multangular cells are completely absent. The cells are noticeably smaller than in fig. 1, B. ×230.

B–D, group F (fixed in alcohol/acetic acid/formalin (B), Bouin's solution (C), and Zenker-formaldehyde (D); celloidin-paraffin embedded after alcoholic dehydration; toluidine blue staining). The cells are noticeably larger than those in fig. 1, A. In fact, in this respect specimens compare favourably with those treated by freezing-drying (fig. 1, B). In all three specimens there is a complete absence of the dark multangular cells. ×230.

was tissue swelling, succeeded by shrinkage after the alcoholic dehydratic preceding embedding.

It would seem that this shrinkage also occurs after procedures other tha alcoholic dehydration. He, however, made no mention of specific tissues cells reacting in different ways to formalin fixation. There is still unexplaine therefore, the selective action of the formalin on a small group of the ce population of spinal ganglia. It might be argued that this formalin-sensitivit implies a structural and functional differentiation of the susceptible cell On the other hand, it is conceivable that a variation in activity level, connotin a particular phase of the cell conversion mechanisms, could influence t immediate effect of formalin fixation subsequently manifest as a qualitativ difference on application of the operative secondary factor.

Caspersson (1950) states that the cytochemical organization of the nerv cell is that of a cell engaged in rapid protein synthesis comparable to an eg cell in a period of growth, and Hydén (1947) reports conspicuous changes i the protein metabolism of adult nerve cells correlated with nerve functio He found evidence of intense activity of the protein-forming system of th relevant cells during sensory stimulation.

It is not suggested here that a protein shift accounts for the formalin sensitivity of particular nerve cells. Groups of spinal ganglion cells ar associated with different sense modalities, and at any given time there wil be a differential stimulation reflected in a diversity of metabolic phase affecting cell mechanisms other than, or in addition to, the cytoplasm protei system.

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The Origin of Sensory Neurones in an Insect, *Rhodnius prolixus* (Hemiptera)

By V. B. WIGGLESWORTH

(*From the Department of Zoology, Cambridge*)

With two plates (figs. 1 and 2)

SUMMARY

Throughout post-embryonic growth in *Rhodnius* new sensory neurones are differentiated from among the ordinary epidermal cells.

The four cells forming the sensillum, the trichogen, tormogen, sense cell, and neurilemma cell probably arise from the division of a single epidermal cell. These four cells are at first alike.

At an early stage in their differentiation, one of them, the sense cell, gives off an axon process which grows inwards, joins the first nerve or axon which it meets, and accompanies this to the central nervous system.

If established sensory nerves are interrupted by burning, the axons grow out from the peripheral end and eventually join up with an existing nerve. Sometimes they grow round and meet the nerve from which they were derived. They may then become trapped outside the basement membrane and grow in loops and circles until a stout nerve is produced which has no connexion with the central nervous system.

At the imaginal moult most of the sensory hairs on the dorsum of the abdomen with their trichogen and tormogen cells disappear, but the sense cells, neurilemma cells, and axons may persist, the distal process of the sense cell ending blindly in the cuticle.

Dermal glands also arise from four cells which at the earliest stage of their development are indistinguishable from the four cells of the sensilla. If the adult is induced to moult the dermal glands may develop non-articulated outgrowths resembling ill-formed hairs.

BENEATH each sensillum in the integument of the insect is a bipolar cell (or group of cells) connected to the sense organ by a distal filament and to the central nervous system by a proximal filament or axon. It is generally believed that this cell is a primary sense cell (vom Rath, 1888; Hanström, 1928), but whether it arises by differentiation from the ordinary ectodermal cells or whether it has migrated outwards from the central nervous system has not been proved.

It was claimed by Vogel (1923) that in Hymenoptera the sensory axon grows outwards from the central nervous system to unite near the periphery with the sense cell; but the centrally placed cell bodies of such sensory nerves have never been recognized. The more usual belief is that the sense cells give off processes which grow inwards (Newton, 1931). Further evidence of this has been given very recently by Henke and Rönsch (1951), Kruminš (1952), and Nüesch (1952).

[Quarterly Journal of Microscopical Science, Vol. 94, part 1, pp. 93–112, March 1953].

Sorokina-Agafonowa (1924), using Dogiel's methylene blue, followed th development of the new sense cells in the legs of *Tenebrio* at metamorphosi She describes a remarkable process by which the existing sense cells wit their distal filaments and axons split repeatedly to give rise to daughter sens cells which eventually became associated secondarily with the developin tactile hairs. Nothing like this has been observed in other insects.

In the post-embryonic development of *Rhodnius* new sensory hairs arise a each larval moult in the spaces between the existing hairs; and after th epidermis has been destroyed by burning over considerable areas of th abdomen, new hairs with normal intervals between them are regenerated i the new integument (Wigglesworth, 1940). The object of the present wor was to use this material for studying the origin and differentiation of th sense cells and their axons.

METHODS

Almost all the observations have been made on whole mounts of the abdominal tergites of *Rhodnius*. The abdomen is cut along the sides and the dorsum dissected off, rinsed in Ringer's solution, and the fat body removed. It is then fixed and stained and mounted entire with the epidermal cells uppermost. In such preparations it is possible to study every cell in the integument (fig. 1, A).

Two methods have been used for staining the nerve axons.

(i) Romanes's method (1950) after fixation in Bouin's aqueous mixture or in Baker's neutral formaldehyde. This gives excellent staining of the axons and the nuclei (fig. 1, B, G) but the cell bodies are usually colourless. It is remarkable that in spite of the intense red-brown staining of the resting nuclei, dividing cells are almost invisible, the chromosomes staining only a very faint grey.

(ii) A modified haematoxylin method. Nerve axons are totally invisible after staining with Ehrlich's or Delafield's haematoxylin; they sometimes

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FIG. 1. A, surface view of epidermis of *Rhodnius* 4th-stage larva at 6 days after feeding. Modified haematoxylin method. (The dark areas are the plaques.)

B, ditto, 3 days after feeding, showing branching sensory nerve. Romanes's method.

C, surface view of epidermis of young adult *Rhodnius*, showing nerve giving off six branches or axons. One axon runs to the sense cell of the campaniform organ above and slightly to the left. The dark lobulated cells are oenocytes. Modified haematoxylin method.

D, campaniform organ showing distal process leading to sharply outlined sense cell with neurilemma cell below. Another elongated neurilemma cell can be seen at the lower part of the figure where the nerve forks. Romanes's method.

E, campaniform organ. The sense cell is at the centre of the figure. A small trachea (out of focus) runs across the distal process. The trichogen and tormogen nuclei appear as a dark crescent above the sense cell; the neurilemma cell as a dark elongated nucleus applied to the lower pole of the sense cell where the axon comes away. Romanes's method.

F, tripolar neurilemma cell where axons unite. Romanes's method.

G, branching nerve showing many separate axons and web-like neurilemma cell with large nucleus at the main fork. Romanes's method.

H, the same.

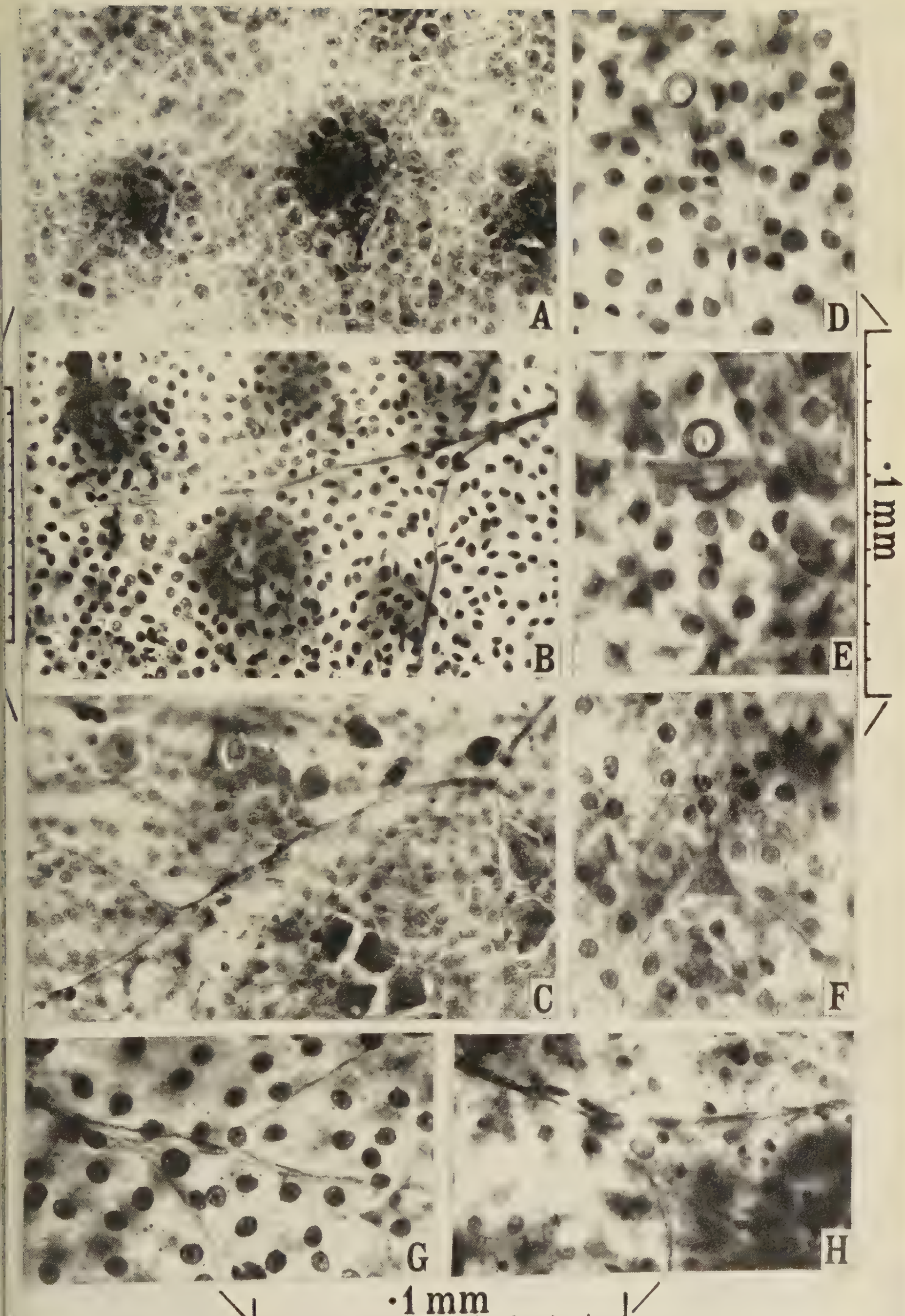

FIG. 1
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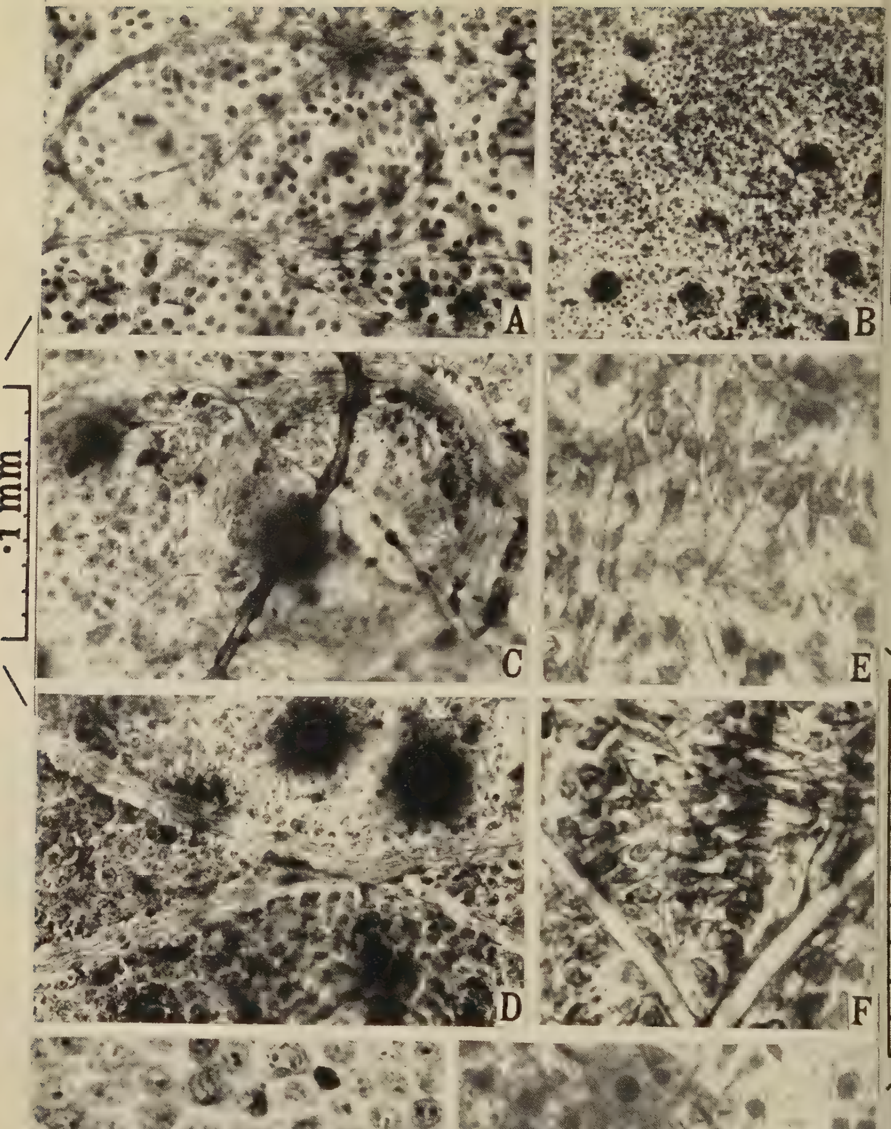


Fig. 2

V. B. WIGGLESWORTH

n faintly in Heidenhain's iron haematoxylin; but the following procedure es much more satisfactory staining.

The tergites are fixed in Bouin, washed for two days in several changes of per cent. alcohol saturated with lithium carbonate, and then placed in al volumes of ethyl alcohol and strong ammonia for a week. This seems have the effect of altering or 'unmasking' the protein of the axon filament rhaps by removing lipoid substances) so that it will now react with iron. e specimen is then placed in saturated iron alum for one minute, washed l in running water, and immersed in dilute ammonium sulphide. If the cimen is mounted at this stage the axons are seen to be stained with iron phide (Wigglesworth 1952*a*); but it is convenient to transfer to potassium ricyanide and, after washing well, to Heidenhain's haematoxylin. The cimen is left in the haematoxylin until the Turnbull's blue has been con- ted to the dark grey haematoxylin lake (usually a minute or two) and is n mounted without differentiation.

Fig. 1, C shows nerves and single axons stained by this method. The staining s not the intensity and clarity of the silver stain but it is less capricious and staining of the cytoplasm serves to reveal the relations between the cells.

nse organs and nerve supply in the integument of the abdomen

There are two kinds of sense organs on the abdominal tergites in the *odnius* larvae: (i) campaniform organs, of which there are about 12–20 on h segment, and (ii) small club-shaped hairs arising from little smooth ques. In the widest part of the abdomen in the 4th-stage larva of *Rhodnius* ere are about 400 of these tactile hairs in each segment.

Fig. 3 shows a small part of a tergite from a 4th-stage larva with the nerves ined with silver. All the club-shaped hairs are innervated; below each of em and below each campaniform organ is a bipolar sense cell with its axon. he axons join neighbouring axons to form small nerves, all these structures ng outside the basement membrane. The terminal nerve shown in fig. 3 ntains a total of twenty-five axons; it was joined by one more axon before finally pierced the basement membrane to enter the body cavity. Some rves are smaller than this, others somewhat larger before they pass out rough the basement membrane.

FIG. 2. A, tangled nerves in the regenerated zone of the epidermis of a 4th-stage larva after rning in the 3rd instar. Romanes's method.

B, a closed 'circular nerve' just inside the unburned zone of a 4th-stage larva after burning the 3rd stage. Romanes's method.

C, part of an elaborate system of circling nerves in 4th-stage larva after burning in 3rd stage. manes's method followed by iron sulphide. A small trachea crosses the field.

D, chiasma of a closed figure of 8 nerve in 4th-stage larva after burning in 3rd stage. manes's method followed by iron sulphide.

E, epidermal cells in adult *Rhodnius* with basement membrane intact, showing anastomosing aments in the plane of the basement membrane. Modified haematoxylin method.

F, the same showing filaments crossing small tracheae.

G, mitosis in epidermis of 4th-stage larva 6 days after feeding. Modified haematoxylin method.

H, surface view of basement membrane at 7 days after feeding, showing haematocytes in ellate form applied to the membrane. Haematoxylin.

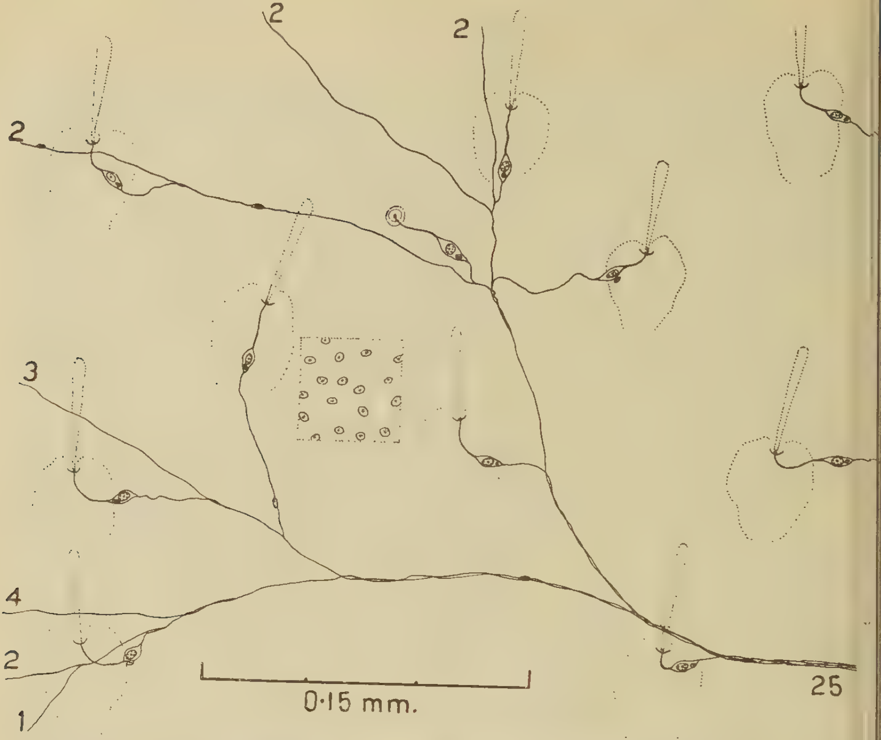


FIG. 3. Small portion of integument of 4th-stage larva one day after feeding showing nerv sense cells, and club-shaped hairs. The figures indicate the number of axons present in ea nerve as calculated from the number of sensilla supplied. The inset shows the distribution epidermal cell nuclei.

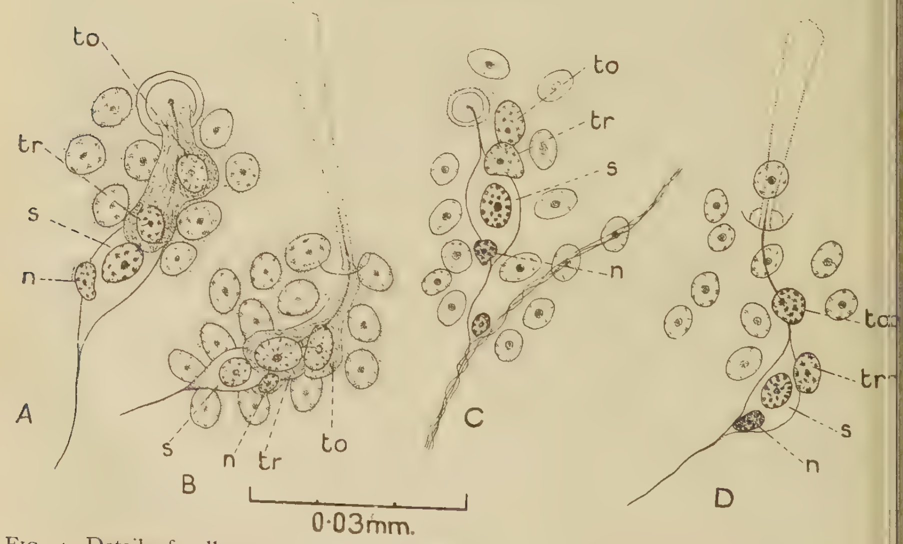


FIG. 4. Detail of cells associated with sensilla in 4th-stage larva as seen in whole moun of the tergites. A, campaniform organ (haematoxylin); B, tactile hair (haematoxylin); campaniform organ (Romanes's method); D, tactile hair (Romanes's method); *n*, neurilemm cell; *s*, sense cell; *to*, tormogen cell; *tr*, trichogen cell.

Fig. 4 shows the detail of these sense organs. There has been much diversity f opinion as to the number of cells associated with the sensory hairs of isects. The best descriptions are those given by Hsü (1938), who agrees with affer (1921) in recognizing four cells: (i) the trichogen cell (*tr*), (ii) the ocket-forming cell or tormogen (*to*) (Wigglesworth, 1933), (iii) the bipolar ense cell (*s*), and (iv) a reniform neurilemma cell (*n*) closely applied to the ense cell. These four cells were recognized in the campaniform organs f *Pediculus* (Wigglesworth, 1941). They are always clearly distinguishable elow the sensilla in *Rhodnius*. They are indicated throughout this paper by ie abbreviations given above. The two cells forming the campaniform organ re doubtless homologous with the trichogen and tormogen (cf. Lees, 1942) ut it is not generally obvious which is which; the labelling of the figures is rbitrary in this respect.

In preparations stained with the haematoxylin method (fig. 4, A, B) the uclei of these cells do not differ greatly from those of the ordinary epidermal ells except that the nucleus of the neurilemma cell is small in size. But after aining by Romanes's method (fig. 4, C, D) the nuclei generally have distinctive haracters. All of them usually stain purple-brown as compared with the red- rown of the ordinary epidermal cells; the nucleus of the sense cell usually as conspicuous blocks of darkly staining chromatin; and the small nucleus f the neurilemma cell often stains almost uniformly black. As already men- oned, the cytoplasm of these cells is stained by haematoxylin, that of the ense cell being usually rather pale. In the silver preparations the cell bodies nd cell boundaries are quite invisible—with the exception of the sense cell, which the unstained cytoplasm is always bounded by a darkly staining ne continuous between the axon and the distal filament. This sharp oundary can be seen in the sense cells supplying the campaniform organs fig. 1, D, E.

All the nerve fibres in the integument of the abdomen terminate in sensilla f these two types; there are no free endings in the intersegmental membranes r elsewhere.

In addition to the neurilemma cell alongside the sense cell, there is a ariable number of neurilemma cells scattered along the nerves. In fig. 3 here are only four of these in the whole system displayed; they have small arkly staining nuclei, usually elongated in the long axis of the nerve. But hey are often far more numerous than this; fig. 5, A shows in detail a branch- ng nerve in which they were particularly numerous. These nuclei often lie vithin the nerve, that is, between the individual axons.

When two axons come together they sometimes proceed as separate fila- nents (fig. 4, C). At other times they appear to form a single filament. Whether hey do really fuse (as was claimed, for example, by Pringle (1938) from the tudy of methylene blue preparations in the cockroach) or whether the individual axons merely adhere together, cannot be affirmed with certainty. In ome preparations (see fig. 5, A, B) a group of axons will appear to fuse but ater separate again, and this may happen several times within a short length

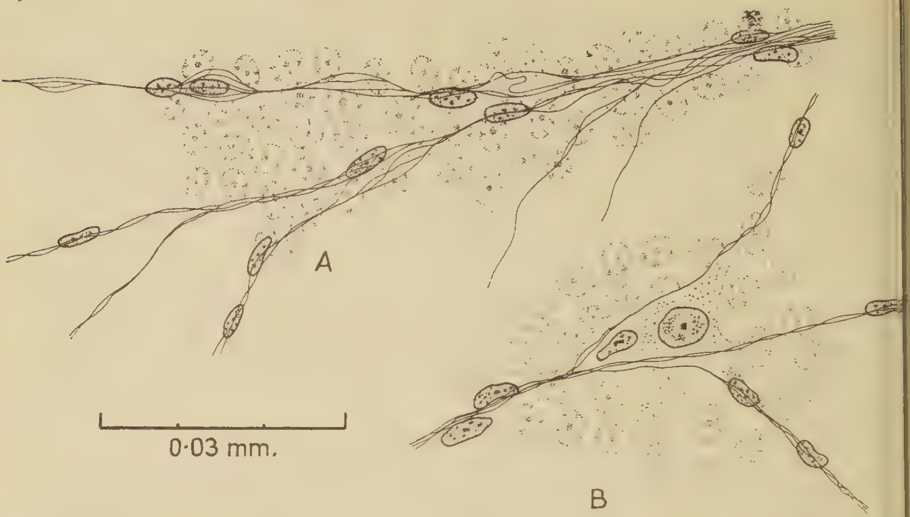


FIG. 5. A, branching nerve in 4th-stage larva 6 days after feeding, showing axons apparen fusing and separating, and numerous elongated nuclei of neurilemma cells; B, web-li neurilemma cell with numerous other neurilemma cells, from the same specimen. Romanes method.

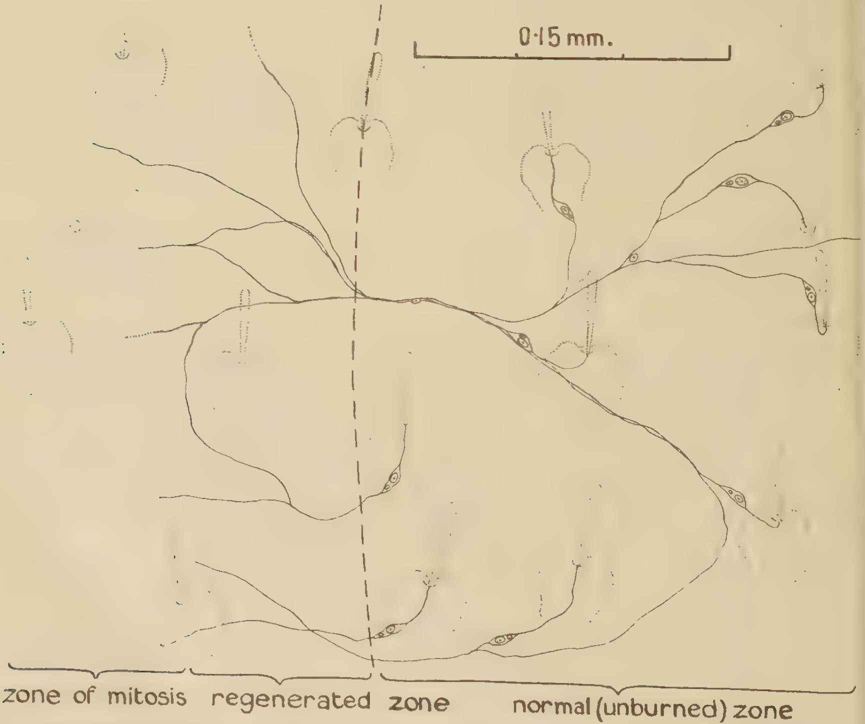


FIG. 6. Nerves in integument of 4th-stage larva 9 days after burning. The broken line mark the limit of the unburned area.

nerve. (A similar adhesion and separation of axons is described by Pantin 952) in the nerve net of Actinozoa.)

Frequently, but not invariably, where two nerves or axons unite there is a iangular cell with a large conspicuous nucleus (fig. 1, F, G, H; fig. 5, B). hese cells often resemble multipolar nerve cells; but I am of the opinion hat they are in fact merely neurilemma cells which have become spread out n web-like form—in the same way as tracheal cells may sometimes be spread ut at the point of origin of a cluster of tracheoles.

egeneration of sensory axons after burning

The epidermis has been destroyed by burning, in the manner previously escribed (Wigglesworth, 1937), by heating the rounded head of an entomoogical pin, dipping it in hot paraffin wax, and moving it to and fro over the rea to be burned. In some experiments almost the whole of one side of the ergites was burned; in others, small groups of ten plaques or thereabouts were olated by circular burns. The burns were applied to 4th-stage larvae at 4 hours after feeding; the tergites were mounted at intervals until the insects ad moulted to the next instar.

Fig. 6 shows a preparation made 9 days after burning. There is a zone of ome 50μ in width at the margin of the burn in which regeneration of the pidermis is more or less complete. Beyond this there is a zone of variable idth in which active mitosis and spreading of epidermal cells is in progress. he nerve axons have grown out into the regenerated zone. At the limit of e burned area, the point to which the nerves had been killed, the axons as ey have grown out have separated and grown in different directions. Often ey have looped round towards the unburned area and in some places have ined up with a nerve coming from this area and, by following it, have been d back again into the uninjured zone.

Fig. 7 shows a much later stage, after the insect had moulted to the 5th nstar. The axons have grown out and looped round in a most irregular maner, sometimes running alone or loosely associated with one or more other xons, sometimes joining to form substantial nerves. Fig. 2, A shows a photoraph of a tangled network of axons of this type. In the specimen shown in g. 7 the growing nerves in this region have eventually discovered two points f escape from the basement membrane as a result of which they never ecame very large.

But sometimes the nerves fail to discover any way of escape from the asement membrane. They remain permanently trapped outside the memrane and appear to go on growing indefinitely. Fig. 8, B shows a circular erve of this type, containing some hundreds of axons all derived from five ense cells. At a low magnification (fig. 8, A) this curious structure appears s a circular nerve lying just inside the unburned area. (The burned area to e right is devoid of plaques and hairs which are not regenerated until the econd moult after an injury (Wigglesworth, 1940).)

Fig. 2, B shows a photograph of a similar small circular nerve. If the insects are kept longer before dissection the nerves become progressively thick Fig. 2, c shows part of an elaborate closed system of circling nerves which places have reached 20μ in diameter and have displaced the epidermal c so that these are crowded along the sides of the nerve. Fig. 2, D shows chiasma of another large nerve which formed a perfect figure of 8 with external connexions.

Fig. 9, B shows the sense cells belonging to a campaniform organ and t

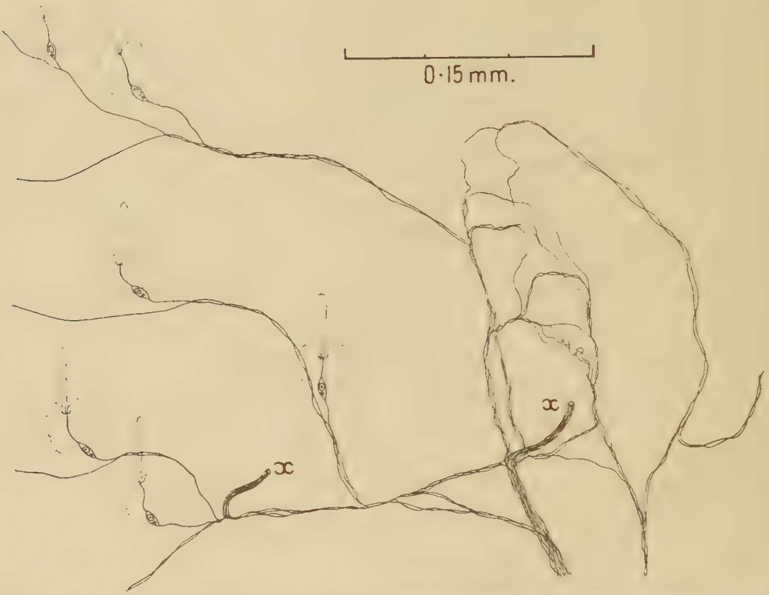


FIG. 7. Nerves in integument of 4th-stage larva after an extensive burn in the 3rd stage. *x* : *x* mark the points at which the nerves have passed through the basement membrane to body cavity.

hairs 9 days after their axons had been interrupted by burning; and fig. 9, shows corresponding organs from the normal area in the same specim where the axons have remained intact. The sense cells with regenerati axons have both nucleus and cell body considerably enlarged and the cy plasm contains small granules staining darkly with haematoxylin.

Moulting and renewal of existing sense organs

The sensory hairs and campaniform organs are renewed at the existi sites at each larval moult and the same four cells are concerned in th formation (Wigglesworth, 1933, 1940). When the old cuticle becomes sep rated from the epidermis, the tormogen and trichogen cells enlarge grea and the new hair grows out through the new socket laid down by the tormog (cf. Wigglesworth, 1933) (fig. 10, B). In preparations stained with silver t

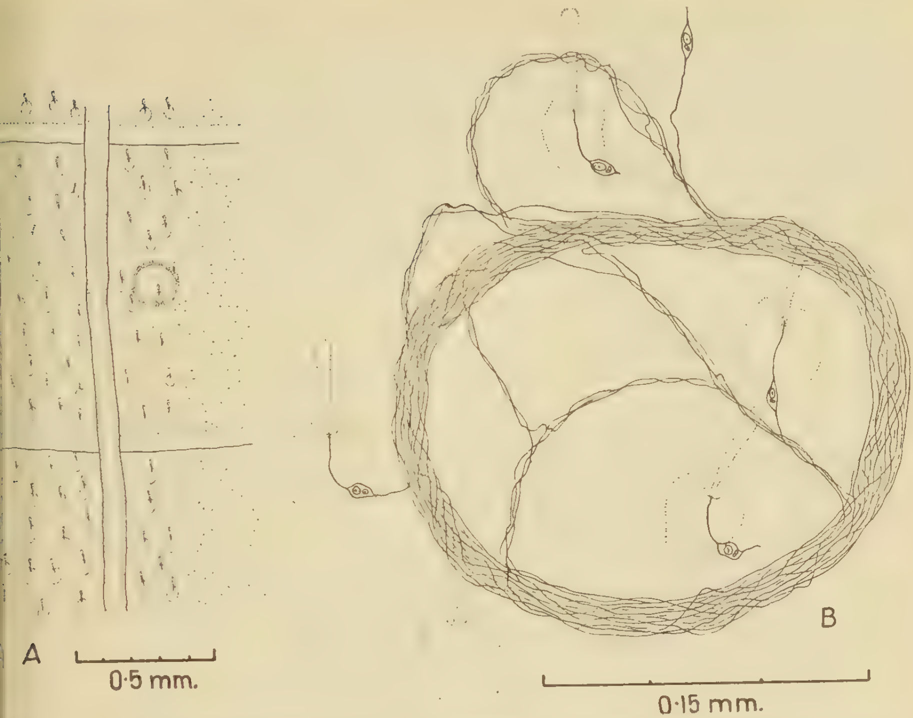


FIG. 8. A, low-power view of integument of 4th-stage larva after extensive burn in 3rd stage. The burned area lies to the right and is devoid of plaques and hairs. A small circular nerve lies just inside the unburned area. B, detail of the 'circular nerve', showing the cell bodies from which it is derived.

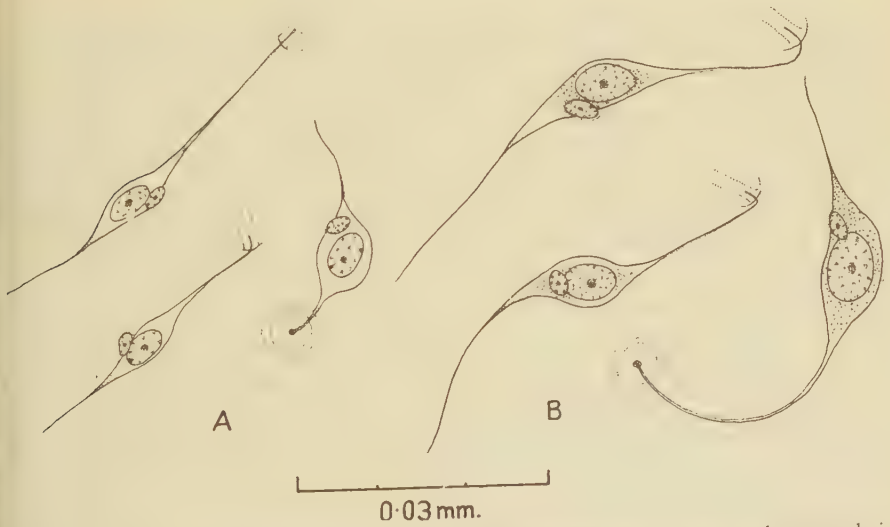


FIG. 9. Sense cells and neurilemma cells belonging to campaniform organs and sensory hairs in integument of 4th-stage larva 9 days after a burn. A, sense cells with intact axons; B, sense cells with axons interrupted by the burn.

newly formed hair stains deep brown, doubtless from the phenolic substan it contains (fig. 10, c).

During this process the distal filament of the sense cell retains its connex with the sensillum on the old cuticle. This may be considerably displac from the position of the new sensillum. The distal filament, staining dee with silver, follows a convoluted course through the moulting fluid betwe the two cuticles. It then passes through the new cuticle at the base of the n hair, or through the centre of the new campaniform organ, and thence to sense cell.

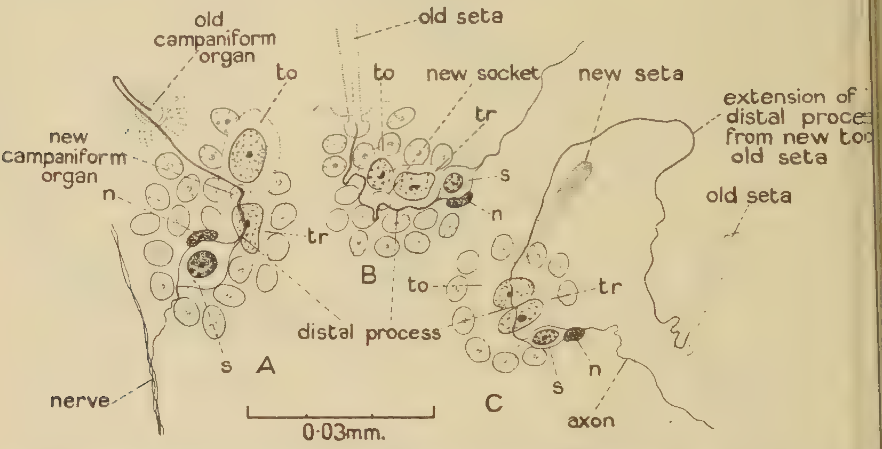


FIG. 10. Stages in the moulting of sensilla in 4th-stage larva (Romanes's method). A, campar form organ; B, hair sensilla at an early stage; C, hair sensilla at advanced stage with new ha almost fully formed. Abbreviations as in fig. 4.

The extension of the distal filament to the old bristle has been described i Lepidoptera (Haffer, 1921), in *Rhodnius* (Wigglesworth, 1933), in termite (Richard, 1952), and in *Psychoda* (Feuerborn, 1927). Feuerborn suggeste that this filament enables the old sense organ to function until an advance stage in moulting. In the 4th-stage larva of *Rhodnius* the epidermis is detache from the old cuticle at least a week before the skin is shed, and in the 5th-stag larva it is separated for almost two weeks. Tests have been carried out on th large tactile spines on the antennae of *Rhodnius* larvae held down by plasticine the sensitivity of the spines to contact with a fine needle being compared i unfed insects and insects at all stages in the moulting process. By this roug test, no diminution in the sensitivity of the spines could be detected up t 24 hours before moulting, which was the latest stage submitted to experiment

Haffer (1921) working with larvae of Lepidoptera and Richard (1952) wit termites observed a period shortly before moulting during which the sensi tivity of the insect is greatly reduced, and they ascribe this to the rupture o the distal filament.

There has been much argument in the past as to whether the campanifor organs are perforated by a pore. It was shown conclusively by Newton (193 that the dome of these organs in the bee is not perforated. These, however, are a new formation in the pupa. Where the organ has already existed in a preceding instar it must be perforated during its development by the distal filament of the sense cell. The opening may none the less be sealed over at the time of moulting by the tanning of the cuticular protein and, in any case, is probably covered by the cement layer (Wigglesworth, 1947). The same will apply to the sealing off of the opening at the base of the sensory hairs.

Fate of sensory hairs at metamorphosis

At the final moult in *Rhodnius* the cuticle of the abdomen is completely changed in character (Wigglesworth, 1933). The plaques have all disappeared and the entire central region of the tergites, where these are covered by the folded wings, is almost devoid of hairs. Some of the campaniform organs persist and may be very large (fig. 11, A). The remaining bristles are changed in form and greatly enlarged but show the usual four cells below them (fig. 11, B).

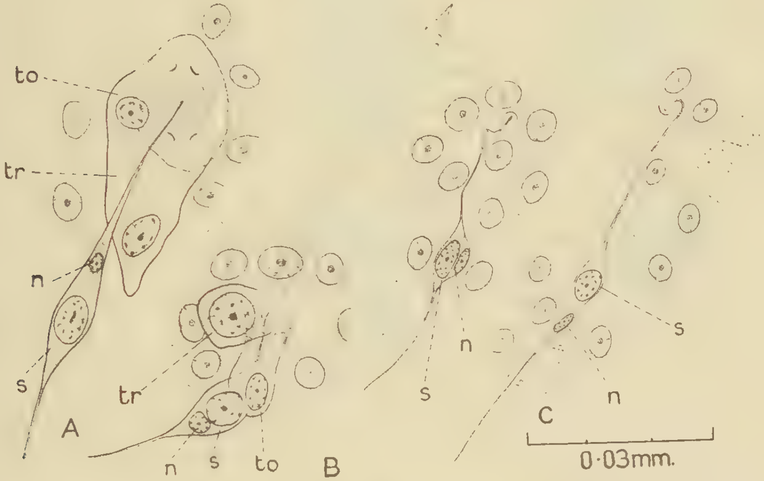


FIG. 11. Sensilla in integument of newly moulted adult. (Modified haematoxylin method.) A, campaniform organ; B, tactile hair; C, sense cells and neurilemma cells belonging to sensilla which have disappeared. Abbreviations as in fig. 4.

We have seen how the sensory hairs remain functional almost up to the time of moulting. This applies equally to the 5th instar: the distal filament penetrates the new cuticle at the sites of the old sensory hairs; but the tormogen and trichogen cells break down and disappear (Wigglesworth, 1933). Consequently, in the newly moulted adult *Rhodnius* there are numerous nerve axons in the epidermis leading to sense cells with their neurilemma cells, and from each of these there runs a distal filament which ends blindly in the cuticle, often below a minute conical projection (fig. 11, C). As in the case of the campaniform organs and other sensilla discussed above, the opening through which the distal filament pierced the new cuticle has been seale over at the time of moulting.

Whether the surviving sense cells and axons serve any function in the adu insect is not known. In specimens mounted one month after moulting, bot sense cells and axons are much more difficult to stain and to find. There no doubt that some of them are still present; it is not possible to say whethe the others have degenerated.

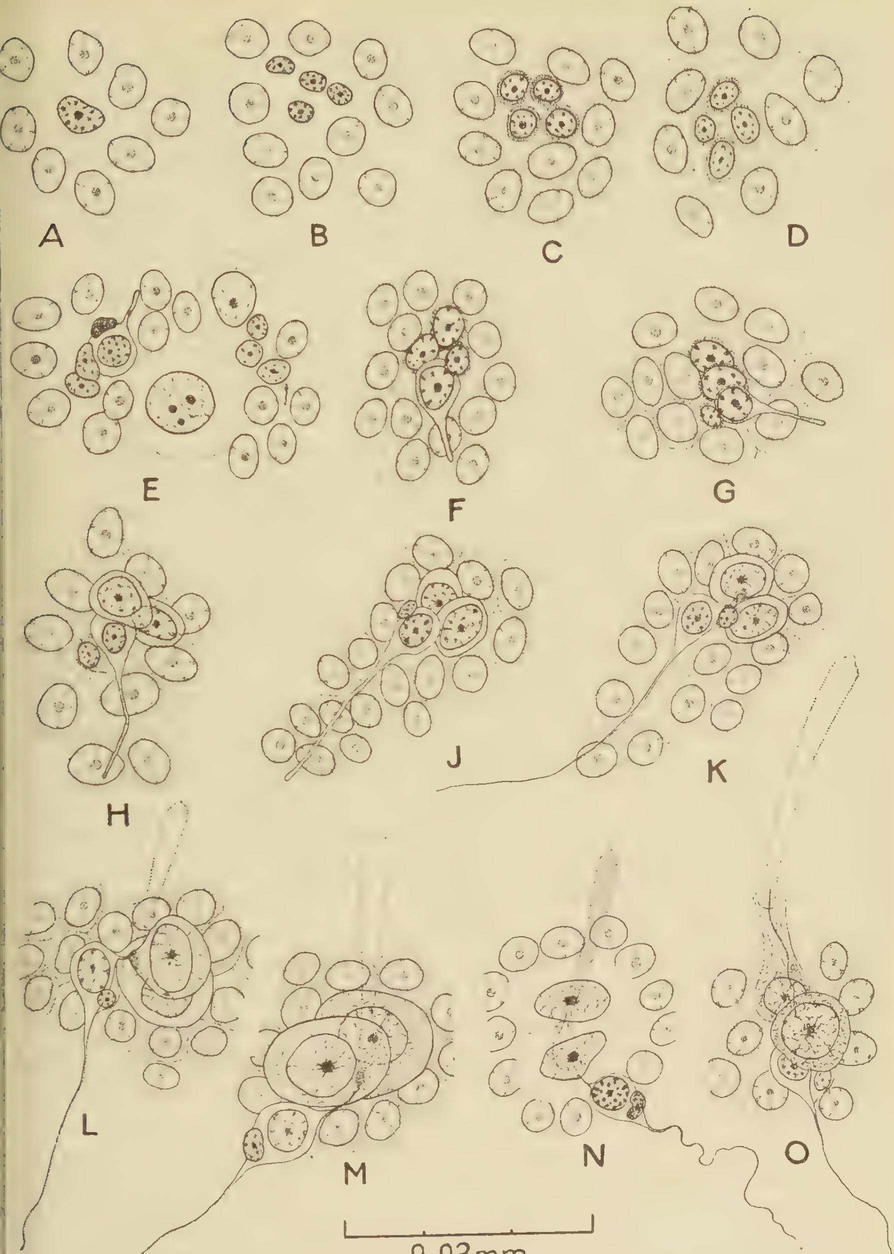
Origin of new sense organs and sensory neurones at moulting

When an extensive area of the epidermis is killed by burning, no ne plaques are differentiated within the regenerated zone at the first succeedin moult; but at the second moult new plaques and hairs make their appearanc at regular intervals over much of the regenerated region (Wigglesworth, 1940 It was thought that such insects would provide good material on which t observe the origin of the new sensilla; but for the demonstration of the earlies stages they have proved disappointing because it is impossible to predict th exact point at which the new organs will arise, and they are therefore ver difficult to recognize among the confusion of dividing cells.

In the normal insect new sensory hairs and plaques are developed at eac larval moult in the spaces between the existing plaques. The hairs increase in number by about 115 per cent. at the first moult, about 50 per cent. at the second and third moult, and about 25–30 per cent. at the fourth moul (Wigglesworth, 1940). The new hairs appear chiefly along the anterior margin of the segments and at those points where the existing plaques are most widely separated. The sites at which the new hairs will appear are therefore to large extent predictable and these points can be closely studied at all stage of moulting. This has proved the most satisfactory method for discovering the earliest stages in the differentiation of new sensilla.

The results are illustrated in fig. 12. The earliest stage recognized with certainty consists of a tetrad of four identical cells with nuclei much smaller than those of the ordinary epidermal cells. In silver-stained preparations (fig. 12, B) these four nuclei show the purple-brown staining which usually characterizes the nuclei of the cellular elements of the sensilla throughout their development. In the haematoxylin preparations (fig. 12, C, D) these nuclei stain a trifle more deeply than the nuclei of the general epidermal cells and they are surrounded by a little darkly staining cytoplasm.

The four cells are presumably the product of rapid division of a single epidermal cell. They appear in the 4th-stage larva at about 6 days after feeding, at the time when active mitosis is in progress in the epidermis (fig. 1, A; fig. 2, G), but it cannot be claimed that their formation has been observed. In silver-stained preparations at this stage one can occasionally observe nuclei, situated at the point where a new sensory hair is expected to appear, which are distinguished from the surrounding epidermal nuclei by the purple-brown colouring already mentioned (fig. 12, A). It is possible to



IG. 12. Stages in the differentiation of hair sensilla in 4th-stage larva. Details in text. (A, B, , and N, Romanes's method, the remainder modified haematoxylin method.) In E the sensilum in process of differentiation lies to the left, a small oenocyte in the middle below, and a dermal gland to the right above.

imagine that these are the mother cells of the tetrads—that they are epiderm cells which have been determined to give rise to sensilla.

The subsequent changes in the form of these cells are quite definite. T nuclei become slightly enlarged (fig. 12, D). Then one of the cells, the o with the largest nucleus, gives out a process which is clearly destined become the axon (fig. 12, F, G). In silver-stained preparations the neurilemm cell can already be recognized at this stage by its small size and dark stainin and its application to the cell, clearly the sense cell, which is giving out t axon process (fig. 12, E).

These changes are occurring very rapidly mostly on the sixth or seven days after feeding, that is, at a time when mitosis is still in progress but gradually coming to an end. Fig. 12, H, J, show further stages in the lengthe ing of the axon filament. Meanwhile the nuclei of the other two cells, destin to become the trichogen and tormogen cells, are enlarging (fig. 12, H, Then the hole in the tormogen through which the hair grows out becom visible, as does the process from the trichogen cell growing into it accom panied by the distal process from the sense cell (fig. 12, K). By this time t axon has usually already met another axon or group of axons and is accom panying these to a sensory nerve.

At this stage, when the process which will form the hair is at the mo active period of its growth, the nuclei of the trichogen and tormogen ce become enormously distended and the cell bodies correspondingly enlarg (fig. 12, L, M). As already noted, in preparations stained with silver at th stage, the newly formed hair stains dark brown (fig. 12, N). Finally, when t hair is fully developed, about 10 days after feeding, the tormogen and trich gen cells and their nuclei are becoming smaller again (fig. 12, O).

It is evident from these observations that sensory neurones are differe tiated from the ordinary epidermal cells throughout post-embryonic growt and that when they first appear they have no connexion with existing nerve How soon the new axon joins up with a nerve, depends upon how close t new sense cell may be to an existing nerve, and how soon the two chance meet. If the new sensillum makes its appearance close to a passing nerv union may be established at a very early stage, before any sign of the ha outgrowth can be seen. The growing axon often follows a somewhat conv luted course. The question whether the axons ever fuse to form a single axo has already been discussed.

The origin of new sensory neurones in *Tenebrio* from the fission of existin neurones as claimed by Sorokina-Agafonowa (1924) has been mentione above. There is no sign of such a process in *Rhodnius*. This author al describes an alternative method of origin in which the sensory neurones a said to be derived from the elongated neurilemma cells of existing nerve This mode of origin would be more difficult to disprove; but nothing ha been seen to suggest that it occurs. Certainly, at the earliest stages of the development the sense cells have no connexion with the nerves.

Nothing definite can be said about the origin of the neurilemma cells alon course of the axons. These, like all the other structures described, are ide the basement membrane and must therefore be derived from the ermal cells or from formative cells of the tracheae which pass through membrane. During the height of mitosis in the epidermis, many more are produced than are needed to form the new integument. Large num- of them break down with the formation of 'chromatic droplets' (Wiggles- th, 1942). It seems likely that some of the excess epidermal cells become ciated with the nerves to form the neurilemma cells.

the formation of scales in the pyralid *Ephestia* as described by Stossberg 8) the scale mother cell first undergoes a mitosis in which the spindle is e or less at right angles to the surface of the epithelium. The inner of the daughter nuclei then degenerates, while the outer undergoes a further osis, in which the spindle is oblique to the surface, to give rise to the scale- ning cell and the socket-forming cell.

he same process occurs during the development of the non-innervated s on the wings of *Limnophilus* (Trichoptera); but in the development of innervated hairs the inner daughter nucleus arising from the first division s not degenerate but persists to form the sense cell which gives off an axon ving towards the base of the wing (Henke and Rönsch, 1951). The sense arises in the same way during the differentiation of bristles in the embryo- development of *Galleria*. In *Galleria*, as in *Rhodnius*, the same formative s persist from one instar to the next, the form of the bristles they lay vn changing at metamorphosis (Krumiņš, 1952).

nparison of sensilla and dermal glands

cattered throughout the integument of the abdomen in the *Rhodnius* larva two sorts of dermal glands (Wigglesworth, 1933). One of these is far more nerous than the other and is responsible for pouring out the 'cement layer' r the surface of the epicuticle at the time of moulting (Wigglesworth, 1947).

s previously described (Wigglesworth, 1933), the dermal glands take ir origin from four epidermal cells, so that at the earliest stages of their elopment they resemble the sensilla. But the new glands arise slightly r in development, and at the time when the tetrads of the new sensilla be seen the new dermal glands have not begun to appear.

ig. 13, A shows a dermal gland at the earliest stage of development. It uld have been indistinguishable from a tetrad destined to form a sensillum that it lay much too close to an existing plaque. As soon as the cells gin to differentiate the dermal gland is very easily distinguished from the sillum (fig. 12, E; fig. 13, B). It consists of one large cell and nucleus, two s with nuclei about equal in size to those of the ordinary epidermal cells, l one with a small nucleus resembling that of a neurilemma cell. The large l becomes the glandular cell; it gradually increases in size and the cyto- sm becomes more dense (fig. 13, C). Later the cytoplasm becomes vacuo- ed (fig. 13, D); and finally the secretion accumulates in a distended vesicular ity (fig. 13, E). There is no nerve supply to the dermal glands.

The dermal glands are most easily seen just before the old cuticle is shed when the distended vesicles are very conspicuous. They occur in groups o four or five around the plaques, with occasional glands in the spaces betwee

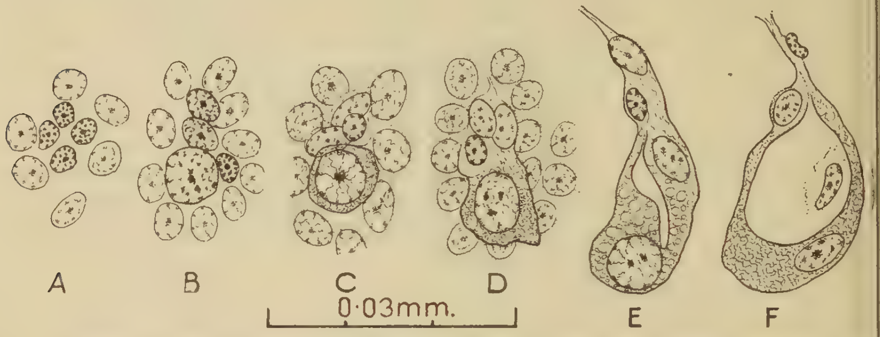


FIG. 13. Stages in the differentiation of dermal gland. (A and B Romanes's method, C— modified haematoxylin method.)

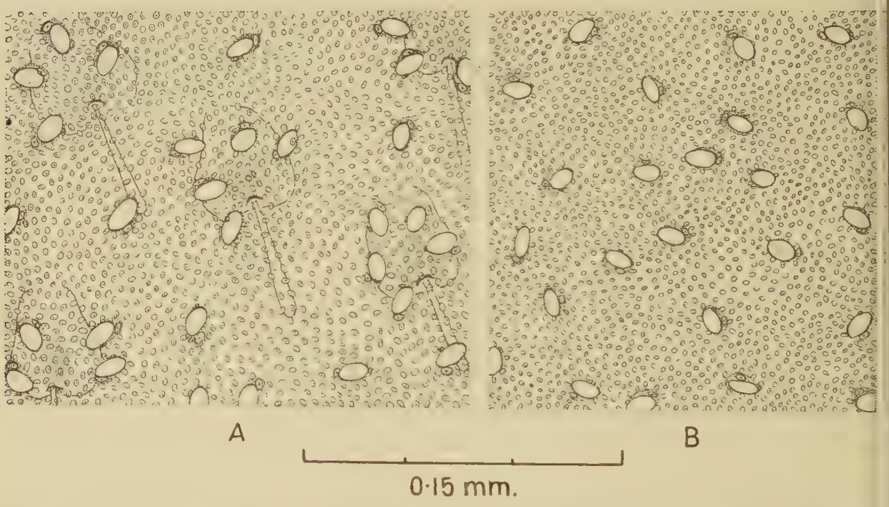


FIG. 14. Epidermis of 4th-stage larva after an extensive burn, mounted shortly before moulting to 5th stage, showing distended dermal glands. A, normal unburned area; B, newly regenerated epidermis over burned area.

(Wigglesworth, 1947) (fig. 14, A). At the first moult after a burn, no sensory hairs or plaques are regenerated on the healed area. But dermal glands are regenerated and they are more or less evenly distributed below the new cuticle (fig. 14, B).

Integration in the epidermis and determination of sensilla and dermal glands

The epidermal cells are separated from the body cavity by a conspicuous basement membrane. This membrane is present in the *Rhodnius* larva before moulting begins, and although it appears to increase somewhat in thickness in the later stages of moulting, this is not suitable material in which to study its origin. As previously described (Wigglesworth, 1933) the haemocytes, which are very scanty in the unfed insect, become exceedingly abundant as moulting proceeds and collect in great numbers on the basement membrane where they spread out in a stellate fashion, and give the appearance of actually contributing to the substance of the membrane (fig. 2, H).

On the outer surface of the membrane the epidermal cells are attached. Their relations are difficult to appreciate from sections (fig. 15, A); but in

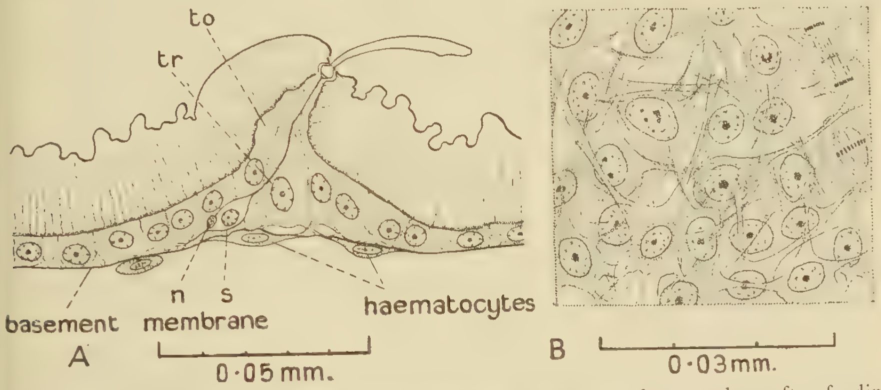


FIG. 15. A, section through cuticle and epidermis of 4th-stage larva 4 days after feeding. B, surface view of epidermis of 4th-stage larva at 6 days after feeding showing anastomosing filaments in the plane of the basement membrane. (Modified haematoxylin method.)

preparations of the tergites mounted flat and examined from below, the epidermal cells are seen to be connected to one another and to the basement membrane by an elaborate meshwork of cell bridges and fibrils (fig. 15, B and fig. 2, E, F). In the paper on wound healing in *Rhodnius* (Wigglesworth, 1937), the impression was formed that it is these epidermal cells which are forming the basement membrane.

Viewed in this way the epidermis evidently forms a syncytium; the cells seem to be independent at their outer extremities but united by filaments at the level of the basement membrane. This union becomes apparent when the epidermal cells migrate during the repair of a wound (Wigglesworth, 1937). (The connexions described as being visible in the resting epidermis (Wigglesworth, 1937) are probably these same filaments, which will lie in the same plane as the nuclei when the epidermal cells become attenuated as they do in the resting state.)

It is within this syncytium that the mother cell of the sensillum is determined. From evidence set out in full in an earlier paper (Wigglesworth, 1940) determination probably occurs soon after the 'critical period'. That is the period when mitosis in the epidermis is rising to its peak. As reported in this paper it is during the later stages of mitosis that the tetrads of the new sensilla appear.

It is probable that these four cells are likewise a syncytium and that as suc they proceed to form the sensillum and the sensory neurone. An intima relation of this kind would explain the inclusion from the outset of the dist process of the sense cell within the substance of the trichogen, and perha the close investment of the sense cell and its axon by the neurilemma cell.

During the healing of a wound or burn the process of general growt leading to moulting is inhibited until the injury is repaired and continuity i the epidermis restored. Moulting then begins but no sensilla are differentiate within the new epidermis. The reason for this is quite unknown. New sensill are regenerated over this area at the second moult and, as described above new dermal glands are regenerated at the first moult. The dermal glands ar developed in just the same way as the sensilla; as we have seen, at thei earliest stages of development they are indistinguishable. There is, therefore no obvious reason why sensilla should not be regenerated at the first moult

These observations suggest that there may be some close connexion betwee the glands and the sensilla and that the factors which lead to their determination are interrelated in some way. Evidence was given in an earlier paper (Wigglesworth, 1940) that the determination of new sensilla is controlled by the distance between existing sensilla as measured by the number of epidermal cells intervening. As a working hypothesis it was suggested that each sensillum and plaque-forming centre drains from the surrounding epidermal cells some substance necessary for their formation or determination. Hence it will be at a point as remote as possible from existing plaques that the new centres will arise.

The even distribution of the new dermal glands regenerated in the region of a burn (fig. 14, B) suggests that the same sort of mechanism is concerned in their determination. The dermal glands arise much closer together than the plaques; they arise at the first moult of the newly regenerated epidermis; and in the normal epidermis they appear for the most part in the form of a ring around each sensory hair. It might be supposed that the same substance necessary for the determination of a sensillum operates in the determination of a dermal gland but that it is effective at a lower threshold concentration.

In the present state of knowledge such a hypothesis must of necessity be vague; but if some common factor of this kind is responsible for the determination of dermal glands and of sensilla, that would suggest that dermal glands and sensilla may be homologous structures. This opinion was put forward by Plotnikov (1904) who claimed that in the larva of *Tenebrio* all intermediate stages between sensilla and dermal glands occur normally in the integument.

That is not so in the normal *Rhodnius*; but if the *Rhodnius* adult is caused to moult experimentally, by implanting in the abdomen the thoracic gland of a moulting larva (Wigglesworth, 1952*b*) or by joining it to a couple of moulting 5th-stage larvae (Wigglesworth, 1940), many of the dermal glands give rise to cuticular outgrowths some of which resemble ill-formed cuticular hairs (fig. 16). These structures are not innervated, nor are they articulated t the base; they are in fact mere extrusions from the cuticle with the duct of he dermal gland opening at the apex; but they do provide a connecting link etween cuticular hairs and glands. Indeed, when they were first observed Wigglesworth, 1940) they were thought for a time to be imperfectly regeneated hairs. They bear a superficial resemblance to the non-articulated sensory pines described by Hanström (1928) in polychaetes.

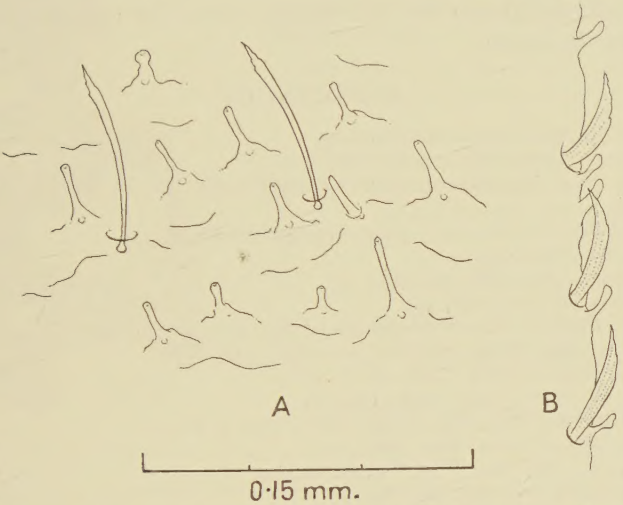


FIG. 16. Cuticle of adult after being induced to moult, showing outgrowths produced by the dermal glands. A, surface view of tergites with two normal hairs; B, margin of abdomen with three hairs.

CONCLUSION

There can be no doubt that the sense cell in the dermal sense organs of *Rhodnius* is a primary sense cell. The fact that when the sensory nerves are interrupted they can be regenerated by the inward growth of axons from these cells, shows that the sense cells are indeed the cell bodies of the sensory neurones. These regenerating axons find their way to the central nervous system by following sensory nerves which they chance to meet. If they fail to encounter such a nerve they will grow in loops and circles and go on growing, apparently indefinitely, without ever regaining connexion with the central nervous system.

The same considerations apply to the new sensilla which arise at each moult. These are derived from four small cells, doubtless the product of a single epidermal cell, which become differentiated to form the trichogen, tormogen, sense cell, and neurilemma cell of the new sensillum. The inwardly growing process from the sense cell forms the axon which joins the first sensory nerve it meets and follows it to the central nervous system. Whether actual fusion of axons ever occurs has not been proved.

It is evident, therefore, that in the insect an important part of the nervous system is being differentiated from the ordinary ectodermal cells through post-embryonic development.

It is interesting that no sensilla should be regenerated in the area of healed burn at the first moult after the injury. They first appear at the seco moult, whereas the dermal glands, which arise in just the same way as sensi from four small cells which become differentiated, are regenerated at the fi moult. This suggests that there is some intimate connexion in developme between the dermal glands and the sensory hairs. The nature of this relatio ship has been discussed.

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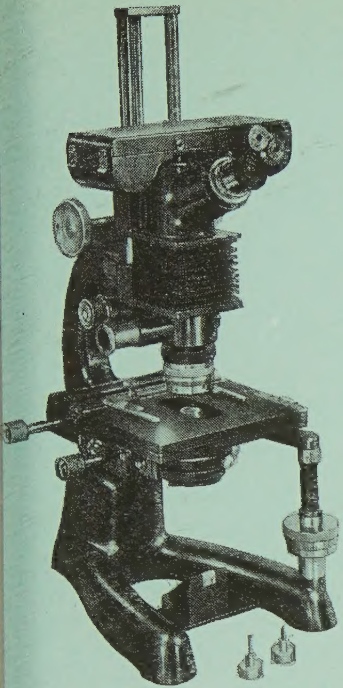

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